

EXPERIMENTAL STUDIES IN THE  
PATHOLOGY OF DIABETES MELLITUS  
WITH PARTICULAR REFERENCE TO  
GLUCAGON AS A SECOND PANCREATIC  
HORMONE.

Experimental Studies in the Pathology of  
Diabetes Mellitus with Particular Reference  
to Glucagon as a Second Pancreatic Hormone.

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SECTION I.

## HISTORICAL REVIEW AND INTRODUCTION

### Early Work.

Shortly after the discovery of insulin by Banting and Best, a number of workers observed that, in addition to the obvious hypoglycaemia produced by injection of this substance, there was an initial hyperglycaemic phase in the blood glucose curve following insulin injection. This phase was of a transitory nature, but quite definite. (Gibbs, Root and Murlin, 1923). It was noticed some time later that this hyperglycaemia would follow the injection of insulin into the portal vein (Collins and Murlin, 1929). Similar observations were made by Bürger and his colleagues working in Germany: (Bürger and Kramer, 1929 a & b.) The factor producing this effect was named glucagon by Murlin.

Insulin was first crystallised by Abel (1926) and it was observed that crystalline insulin did not produce this effect, but that it was caused by an impurity in the residue after the insulin had been crystallised. (Geiling and de Lawder, 1930; Bürger and Kramer, 1930).

After this, interest in the subject subsided though Bürger and his colleagues continued to study the problem (Bürger, 1930), (Bürger and Brandt, 1935), (Bürger, 1937).

Lipocaic.

Dragstedt described some experiments in which he had administered an alcoholic extract of beef pancreas to depancreatized dogs (Dragstedt et alii, 1936). He claimed this substance permitted survival and prevented and relieved the fatty degeneration and fatty infiltration present in the livers of such animals. He called the substance producing this effect lipocaic and believed it to be a new hormone concerned in the transport and utilization of fat.

Bensley and Woerner (1938) claimed to have prepared an extract of pancreatic alpha cells (A-cells) which they gave by continuous intravenous infusion to guinea pigs. Their method of extraction was extremely crude and undoubtedly the extract contained a variety of factors. It produced an increase in the liver fat of the recipient guinea pig and a decrease of liver glycogen as well as some histological evidence to suggest that when glucose was given with this extract, the beta cells (B-cells) actively secreted and the A-cells became loaded with granules which was taken to indicate an inhibition of secretion. These authors maintained that the A-cells could be extracted only in aqueous solution. They identified their extract with

that of Dragstedt although lipocaic was extracted with alcohol. However, it seems possible in view of the glycogenolytic effect, that some glucagon may have been extracted, although the B-cell exhaustion was probably produced by hyperglycaemia.

The existence of lipocaic and the effects claimed for it by Dragstedt were not confirmed by other workers (Best and Ridout, 1938), who considered that no new specific factor had been demonstrated, and that the properties of a sample of lipocaic prepared by Dragstedt and his collaborators were only those which might have been expected from its choline and protein content.

In a further series of experiments, Dragstedt and his collaborators observed that lipocaic had no ketogenic effect on depancreatized dogs (Dragstedt et alii, 1941). In this work it was noted that during the period of administration of this substance to depancreatized dogs that the urinary sugar excretion rose from 3 to 30 grams/day. It is supposed, however, that even if a hyperglycaemic substance were present in lipocaic that such an extract given by mouth would very probably improve intestinal absorption of carbohydrate and thus increase glycosuria.

Effects of Pancreatectomy on Insulin Requirements.

Marks and Young (1939), in a paper which is a model of accurate scientific observation, published some facts of which the full significance was not appreciated for some years. They noticed that some of their pituitary diabetic dogs required more insulin than did depancreatectomized dogs. Further, that removal of the pancreas from a pituitary diabetic dog resulted in "a slight, but possibly not significant fall in insulin requirement." This observation was the first of many of a similar nature noting this difference and suggesting that diabetes was not necessarily an entirely B-cell deficiency, but that an anti-insulin influence had been removed by pancreatectomy, which thus alleviates the diabetes to some extent. This was a most interesting result when exactly the opposite might have been expected.

These findings were confirmed some years later by Dragstedt et alii (1943). Dragstedt noted that a 95% depancreatectomized dog required definitely more insulin than a completely depancreatectomized animal. The authors had at that time two alloxan diabetic dogs stabilized on 32 - 36 units of soluble insulin which they considered was more than would be required by a depancreatectomized dog. They very properly



pointed out that the better digestion and absorption of the animals with pancreatic remnants was undoubtedly a factor in their greater insulin tolerance, but that this might not be the whole explanation.

Corroborating evidence was coming in from clinicians also. Rockey (1943) performed a total pancreatectomy for carcinoma, and the man survived the operation for 15 days. During this period the patient received 150 G. of glucose/day and his glycosuria was controlled on 27 units of insulin. Discussing this case Mirsky (1945) asked - "Why should a man sometimes require more insulin than his pancreas is apparently capable of producing?" He speculated on the possibility of the pancreas secreting an anti-insulin hormone, but thought that pancreatic extracts exercising such an anti-insulin effect might contain proteinases which were capable of destroying insulin. He suggests also that insulin may stimulate production of an insulin antibody.

Two cases were reported of pancreatectomy performed on diabetic patients - Brunschwig et alii (1945) and Dixon et alii (1946). In Brunschwig's case the pre-operative insulin dosage was 40-65 units/day on a diet of

carbohydrate	398 G.	Body weight was 72 Kg.
protein	200 G.	
fat	12 G. - glucose equivalent 511 G.	



Two months after operation the figures were:

insulin 40 units/day on a diet of

carbohydrate	401 G.	
protein	102 G.	glucose equivalent 459 G.
fat	11 G.	

Body weight was 59 Kg.

The authors comment that the reduction of insulin dosage may have been due to diminished body weight or diminished food intake, but the striking fact is that the insulin consumption was not markedly increased, and so they conclude that an extra-insular factor must contribute to the diabetic syndrome. Similar findings were observed in Dixon's case, although the insulin consumption here was a little increased. These patients suffered from a marked insulin sensitivity after pancreatectomy, and such patients have died of insulin sensitivity reactions (Gaston 1948). Thorogood and Zimmermann (1945), in a major investigation, performed pancreatectomy on alloxan diabetic dogs measuring their insulin requirements before and after. They found that alloxan diabetic dogs required more insulin before pancreatectomy than after, but that they were more stable and less likely to go into coma before than after the operation. They considered that the simplest explanation of this phenomenon was to postulate the existence of a second endocrine factor in the pancreas, hyperglycaemic in its effect, yet preventing ketosis. Realising

the fallacy inherent in comparing insulin requirement before and after pancreatectomy due to removal of digestive ferments, Roderiguez-Candela et alii (1947) undertook the most difficult task of determining the insulin requirement and then ligating the pancreatic ducts of alloxan-diabetic dogs, allowing the pancreas to fibrose and atrophy, and then estimating the insulin requirement of the animal. Pancreatectomy was now performed and the insulin requirements again determined. They found that ligation of the ducts made no difference to the requirements of insulin, but that subsequent removal of the pancreas caused a 20-50% decrease in insulin requirement. From these facts they suggest the strong possibility that the A-cells of the pancreas may secrete an anti-insulin factor or hormone.

These views have been contested by Mirsky et alii (1951) who state that, whilst they agree that a diminution of glycosuria occurs in an alloxan diabetic dog following pancreatectomy when it is maintained on a constant dietary intake and insulin dosage, the same does not hold if the comparison is made on fasting dogs. In the latter case the insulin requirement is greater after pancreatectomy. They maintain that there is considerable malabsorption of protein and of carbohydrate following pancreatectomy and they consider that the diminished

glycosuria in an alloxan diabetic dog following pancreatectomy is due simply to failure to absorb carbohydrate. Mirsky and his colleagues do not accept that a hyperglycaemic, glycogenolytic factor plays a rôle in the genesis and metabolic derangement characteristic of diabetes mellitus.

Roderiguez-Candela (1952) points out in answer to the above views that the ligation of the pancreatic ducts would have produced an absence of pancreatic enzymes both before and after pancreatectomy.

Lukens (1952) raises the point as to how effective was the duct ligation. However, the general weight of evidence tends to indicate at least that the insulin requirements after pancreatectomy are considerably less than might reasonably be expected and that certainly the possibility of a second pancreatic islet-cell hormone exists. This opinion is supported by Young (1949) in the Jacobaeus lecture of that year. "It is possible that the greater insulin requirements of a metahypophyseal diabetic dog (i.e. a permanent pituitary diabetic or alloxan diabetic dog) as opposed to a depancreatectomized one is due to the presence of A-cell rests in the islets: the acinar tissues of the pancreas or the A-cells of the pancreatic islets possibly secrete a substance which acts antagonistically

to insulin". He further speculates on the possible identity of this substance with the glycogenolytic factor of Sutherland and de Duve (1948). (Vide infra).

Glycogenolytic and Hyperglycaemic Actions of Insulin and General Properties of Glucagon.

Bridge (1938) noted that the effect of insulin (Lilly) on glycogen reserves was a shift in glycogen deposition from liver to muscle tissue. This revived controversy as to whether insulin had a glycogenolytic action on the liver. Discussing this problem de Duve (1945) said he considered that insulin might have a double action on the liver causing both glycogen synthesis and glycogenolysis.

Subsequently, de Duve, Hers and Bouckaert (1946) reported some experiments in which a comparison was made between the hyperglycaemic effects of the Lilly insulin used by Bridge (1938) and the lack of such an effect in the Novo insulin produced in Copenhagen. They considered that an impurity was present in the Lilly insulin, hyperglycaemic and glycogenolytic in its effects. They considered that glycogenolysis is not a property of the insulin itself. These authors stated (de Duve and Hers, 1947) that their experiments showed that insulin favoured glycogen

synthesis and that the glycogenolytic effect produced by certain insulins was probably due to (1) the effect of adrenaline consequent on the hypoglycaemia produced by the insulin, and (2) the presence of a hyperglycaemic impurity. Olsen and Klein (1947) found the same effects when comparing Lilly and Novo insulin and also found that Sharpe and Dohme, and Squibb insulin also carried the hyperglycaemic impurity. They observed that this effect occurred only after intravenous injection, but not after intramuscular injection into cats.

In a detailed study Zimmermann and Donovan (1948) found that the hyperglycaemic effect persisted after inactivation of both amorphous and crystalline insulins with cysteine. They found only a small hyperglycaemic effect on depancreatized dogs and observed that it did not prevent ketosis after a dose of glucose in such animals, nor did the inactivated insulin protect mice against insulin convulsions. They conclude that the hyperglycaemic impurity acts only through its effect on hepatic glycogen levels. They raise the question of its identity with the hyperglycaemic, glycogenolytic factor extracted from pancreas by Sutherland and de Duve (1948). Finally, they state their doubts at that time as to whether the hyperglycaemic principle can yet



be considered to have a normal function as an internal secretion of the pancreas.

The foregoing observations were confirmed by Sutherland and Cori (1948) who assayed the hyperglycaemic factor of insulin on sliced liver, observing the amount of glycogenolysis produced. They found that the factor, which they called hyperglycaemic, glycogenolytic factor or H.G.F., acted on the phosphorylase system of the liver.

Sutherland and de Duve (1948) prepared an extract of dog pancreas by a method similar to that used for manufacturing insulin which they found to be glycogenolytic when assayed on liver slices. They obtained this factor also from the gastric mucosa of dogs, but it could not be demonstrated in any other tissue or in the exocrine secretion of the digestive tract. The factor was present in large amounts in foetal calf pancreas and in the tail of the pancreas - both places where islet population is very dense. It could be extracted in large amounts from ligated, sclerosed pancreas. It was obtained from extracts of alloxan diabetic pancreas. The latter extracts produced prolonged hyperglycaemia, when given intravenously to rabbits, without subsequent hypoglycaemia.

These results were suggestive of an A-cell origin of this factor as in alloxan diabetes the

B-cells would be largely destroyed.

These workers remarked that the curious combination of islet tissue and gastric mucosa of dog as a source for H.G.F. closely paralleled the distribution of argentophil cells in the fundus of dog stomach (Tehver, 1930) and the argentophil cells of the pancreatic islets noted by Ferner (1938). The latter using the Gros-Schultze method considered the silver staining cells to be identical with the A-cells. Ferner considered at that time that the A-cells on the periphery of the islets were immature non-functioning cells and the B-cells in the centre were the mature ones. He found that these cells were a great deal more numerous in children and could be seen only occasionally in adults. He pointed out that the presence or absence of granules in the A-cells depended on the time after death that fixation took place.

Van Campenhout (1933) had also noted the presence of silver-stained cells in the pancreatic islets of man and also of calf, pig and chick. He also noted similar cells lying in relation to the acini and pancreatic ducts and shrewdly observed that he thought they were precursors of the islet cells.

However, opinions as to the function of

these cells were not uniform. Jacobson (1939) considered the ones found in the stomach might be the source of the anti-anaemic principle and absence of these cells was noted in cases of pernicious anaemia. Much significance has been attached by these authors to what is, after all, not a true histochemical stain, but a rather variable physical deposition of silver salts.

To end their important contribution Sutherland and de Duve (1948) state that "while it is tempting to conclude that the glycogenolytic factor is a new hormone of the pancreas, it seems unwarranted in the absence of a clear cut demonstration that it is secreted into the blood stream and that it participates in the regulation of the blood sugar level".

The properties of this hyperglycaemic factor have been investigated by Sutherland (1950) who carefully distinguished its effects from that of epinephrine pointing out that although both substances cause glycogenolysis in the liver only epinephrine raises the blood lactic acid. Sutherland also again noted that the action was closely related to the phosphorylase system of the liver and that it could not take place in frozen or homogenized liver, or in other words, an intact cellular structure was necessary for its action.



Vuylsteke, de Duve and Nys (1950) reported that glucagon was active subcutaneously as well as intravenously and that it does not affect the blood pressure or the heart as does adrenaline. They confirmed Sutherland's (1950) finding on the difference between the blood lactic acid after adrenaline and glucagon injections. They also noted that a fall of blood eosinophils was produced by glucagon (glucagon is generally called H.G.F. or "le facteur H-G" in the Belgian literature). The chemical properties of glucagon have been described by de Duve (1951). It has the general properties of a protein or large polypeptide. It is not dialysable and it is not inactivated by treatment with mild alkali or cysteine.

Discussing the work of de Duve and of Sutherland and Cori in a Banting memorial lecture Young (1950) says that there is no direct evidence that this A-cell substance is indeed liberated into the blood stream and even if it is, the available evidence suggests that it would not raise the insulin requirements of the depancreatectomized human being by more than 20 units/day. The glycogenolytic properties of glucagon were confirmed by Pincus (1950) who added the information that hyperglycaemia could not be produced in the assaying animal by the extracts of pancreas after hepatectomy or when hepatic

glycogen was low such as in uncontrolled diabetes or starvation. He found also, as had Collins and Murlin (1929), that it was most effective when injected into the portal vein.

Gaede, Ferner and Kastrup (1950) claim to have made a pure extract of insulin-free glucagon from horse and cattle pancreas which they find consistently hyperglycaemic when injected into puppies. They obtained this substance also from the pancreas of alloxan diabetic puppies incidentally confirming the work of Sutherland and de Duve (1948). They also extracted glucagon from the pancreas of puppies, which had their pancreatic ducts ligated for three months and were then given alloxan. However, an extract obtained by such a method cannot be regarded as chemically pure or even insulin free, as a little insulin producing B-cell tissue would almost certainly remain even after the administration of alloxan. They thought glucagon acted with insulin to regulate carbohydrate metabolism. It is not yet generally accepted, however, that glucagon has such a significant rôle to play. (Young, 1950).

Attempts to show direct secretion of H.G.F. into the blood.

An attempt to provide direct evidence

of secretion has been made by Foa et alii (1949). In a considerable number of dogs these workers anastomosed the pancreatiko-duodenal vein or the mesenteric vein of a donor dog A. with the femoral vein of a recipient dog B. A limited return flow of blood was secured by anastomosing the femoral artery of dog B. and the femoral vein of dog A. They found that the blood sugar of a dog receiving pancreatic blood from a normal donor injected with glucose decreases sharply - presumably due to the hyperglycaemic stimulus to insulin secretion. Blood from the mesenteric vein in this experiment produced hyperglycaemia in dog B. They found that if the anastomosis is made from the pancreatic vein of an alloxan diabetic dog to a normal donor a marked hyperglycaemia results in the recipient. They suggest that this is evidence of the alloxan resistant part of the pancreas secreting a hyperglycaemic substance. This is of course true, but the exocrine effect of the pancreas is not eliminated in this experiment.

An attempt to show direct secretion into the blood stream has been made in Italy by Cavallero and Malandra (1950 a & b) using pancreatic grafts placed intraperitoneally and subcutaneously in rats. They grafted (1) normal rats with normal rat pancreas; (2) normal rats with alloxan diabetic rat pancreas;

(3) partially  
depancreatectomized rats with alloxan diabetic  
rat pancreas.

They claimed the results of these experiments show that there is in the pancreas a hyperglycaemic, glycogenolytic substance which perhaps comes from the A-cells. Their table III (Cavallero and Malandra, 1950 b.) shows a moderate hyperglycaemia after grafting normal rats with alloxan diabetic pancreas, and their table VI indicates an increase of glycosuria in partially depancreatectomized rats after grafting with an alloxan diabetic pancreas and in normal rats after such a graft, but to a lesser extent. Table V shows the rather surprising fact that transplantation of alloxan diabetic pancreas into a partially depancreatectomized rat makes no appreciable difference to the blood sugar which one might have expected if functioning A-cells were present in the grafts. Their other results do not appear to show any significant difference produced by the alloxan diabetic grafts or the normal grafts in response to a dose of insulin or of glucose. These experiments are also open to the same criticism as that of Foa et alii (1949) that the exocrine portion of the grafts is still functioning.

Nevertheless, though not conclusive, these

experiments are of considerable interest. An experiment done by Bornstein, Reid and Young (1951) provides some rather more definite evidence of the direct secretion of glucagon by the pancreas into the blood stream. These workers prepared some alloxan diabetic, hypophysectomized, adrenalectomized rats (A.D.H.A. rats) by the method of Bornstein (1950). These rats were regarded as very sensitive indicators of carbohydrate metabolism being capable of detecting extremely small quantities of injected insulin as noted by variations in their blood sugar.

Pituitary growth hormone was given to such A.D.H.A. rats and to intact cats under such conditions that diabetes was induced. Portal blood from these animals was consistently found to exert a hyperglycaemic action when given to a recipient A.D.H.A. rat. Such a hyperglycaemic effect was not found when growth hormone was administered directly to the recipient rats. The authors considered these experiments were consistent with the view that under the influence of growth hormone the pancreatic islets liberate their contained hyperglycaemic substance into the portal blood. They point out that H.G.F. was not detected in blood from the femoral vein.

This contribution is of considerable significance in that it was the best demonstration



then available that the pancreas, and probably the A-cells of the pancreas, secreted a hyperglycaemic substance into the portal blood. It suggested in addition that the pancreatic islets might secrete this substance as a direct result of anterior pituitary growth hormone stimulation. These observations were in accord with the findings of Anderson and Long (1948) who removed rat pancreas and put it in a perfusion apparatus. When they put a high glucose concentration in the perfusing fluid the perfused pancreas responded by secreting insulin which was detected by estimating the blood sugar levels on adrenalectomized hypophysectomized rats injected with the perfusate. However, when growth hormone was added to the high glucose-containing perfusing fluid no insulin was secreted, but when the growth hormone was inactivated the perfused pancreas secreted insulin, responding in the normal way to the high glucose stimulus. This suggested that growth hormone either inhibits insulin secretion or causes an anti-insulin factor to be secreted.

#### Influence of anterior pituitary gland on Pancreatic Islets.

Concerning these last two experiments (Bornstein, Reid and Young, 1951), (Anderson and Long, 1948) it is appropriate to mention briefly

the diabetogenic effect of anterior pituitary extracts and growth hormone, though it is not proposed to discuss this problem in detail. The reader is referred to the undermentioned papers for further information on this subject which is relevant to this thesis only so far as it concerns glucagon secretion.

Numerous authors have described the diabetogenic effects of anterior pituitary extracts when administered to a variety of animals (Young, 1937), (Houssay et alii, 1942), (Ogilvie, 1944). The whole subject of the relationship of the anterior pituitary gland to diabetes has been reviewed by Young (1949). The histological appearances of the pancreas in anterior pituitary diabetes have been described by Richardson and Young (1938). They say there are three possible responses to anterior pituitary extract:

- (1) Proliferation of B-cells;
- (2) Hydrops of B-cells, which was thought to be due to exhaustion following prolonged hyperglycaemia;
- (3) Disappearance of B-cell granules.

Finally, hyalinization may occur. Effects on the A-cells are not described. Ogilvie (1949) described proliferation of the islets and the appearances of islet regeneration from ductules following the administration of anterior pituitary

extract (A.P.E.) to alloxan diabetic rabbits.

Best, Campbell and Haist (1939) note that the administration of A.P.E. to a dog results in a diminution of insulin content in the pancreas.

Young (1941) observed that puppies given A.P.E. respond by growth and not by becoming diabetic as does an adult dog. After some months one of his puppies ceased to grow and then became diabetic. Insulin given with the A.P.E. induces such a puppy to resume growth (Young, 1944). Similar effects can be produced by pure growth hormone which Young (1949) considers to be identical with the diabetogenic hormone, producing diabetes in adults and growth in the young. The effect of growth hormone in producing hyperglycaemia in cats is described in an excellent paper by Cotes, Reid and Young (1949). The high incidence of diabetes in acromegalics has been noted in connection with the possible place of excess secretion of growth hormone as a cause of diabetes (Young, 1950).

Bornstein and Lawrence (1951) describe injecting the plasma, from two middle-aged acromegalics with a blood sugar of 300-400 mgm.% and heavy glycosuria, into A.D.H.A. rats. It produced a rise in the blood sugar of these animals and was the only diabetic plasma to



produce such an effect in their numerous assays of plasma for insulin in different types of diabetics. Bornstein has found, using this method of assaying plasma insulin on A.D.H.A. rats that the younger, more severe type of diabetic has no plasma insulin and the middle-aged, obese type has free insulin (Bornstein and Lawrence, 1951), (Bornstein and Trehella, 1950). Further, it was noted that the plasma from insulin resistant diabetics would, if injected into A.D.H.A. rats make them insulin resistant also.

It is suggested by Bornstein (1951) that in the type of diabetic who carries no free insulin in the blood that this may be due

- (1) To a failure to secrete insulin; or
- (2) To utilization in excess of the maximum secreting rate of the pancreas; or
- (3) To destruction of insulin at a pathological rate; or
- (4) To an excess of substances which raise the blood sugar.

Milman and Russell (1949) found that growth hormone, contrary to the findings of the previous workers, depressed the blood sugar of intact rats; but raised the blood sugar of depancreatized and alloxan diabetic rats. They interpreted these results as suggesting that growth hormone in intact animals would stimulate insulin

secretion whereas if islet tissue is lacking hyperglycaemic or contra insulin effects are seen.

From the available evidence it is thus seen that the exact rôle of growth hormone in the production of diabetes mellitus is still controversial, but there is evidence to suggest that in acromegalic diabetes a hyperglycaemic substance is present in the blood. Experimentally it is found that growth hormone produces hyperglycaemia according to the majority of authors, and given for a longer time causes destruction of the islet B-cells. Extreme views are held by some authors (Ferner, 1951) suggesting that growth hormone is an alphacytotropic factor, and that the relationship between A and B cells of the islets determines the stability of carbohydrate metabolism, and that disorder of such a normal relationship can lead to insulin resistance, and can explain the causation of all types of clinical and experimental diabetes, but these views probably are an oversimplification and are only partly accepted. It is more probable that growth hormone stimulates growth of both A and B cells (de Duve, 1953).

No doubt, however, the possibility of such a relationship between the growth hormone of the anterior pituitary and the pancreatic A-cells, was given due weight by Bornstein, Reid and Young when they gave growth hormone to produce hyperglycaemia in the A.D.H.A. rat (Bornstein, Reid

and Young, 1951).

Alpha-cell damaging substances.

Since the work for this thesis was begun, de Duve (1953), in his admirable review of the whole subject of glucagon, draws attention to work done by van Camphenout and Cornelis (1951 a & b). These Belgian workers injected cobalt chloride subcutaneously into guinea pigs and found a hydropic degeneration of the pancreatic A-cells was produced attended by loss of granules, and that there was no histological evidence of B-cell damage. They also gave cobalt by intra-cardiac injection and found a hyperglycaemia of 200 mgm.% and 286 mgm.% was produced. They suggest that there is first an excitation and then a degeneration of the A-cells.

Subsequently, Vuylsteke, Cornelis and de Duve (1951) estimated the glycogenolytic effects of pancreatic extracts from the guinea pig after a 6-day course of cobalt and found a diminution of glycogenolytic activity of the order of 60%. These results are extremely interesting and adduce more evidence of the A-cells being a probable source of glucagon. Davis (1952) has produced similar histological effects on the A-cells with Synthalin A. The effects of this latter substance are only temporary as are those

of cobalt chloride, but are associated with hypoglycemia.

De Duve (1953) summarizes his views at that date on glucagon - "The evidence shows that the pancreas contains a highly active and specific glycogenolytic polypeptide, which originates in the alpha cells of the islets and can act as a powerful insulin antagonist; and that an alloxan-resistant part of the pancreas, probably not the acinar tissue, secretes into the blood-stream a substance which raises the blood sugar level, lowers the glycogen content of the liver and decreases the effectiveness of insulin. The logical inference is that we are dealing with one and the same substance - in other words, that glucagon is the alpha cell hormone whose existence is revealed by the physiopathological data - but it must be admitted that this has not been proved."<sup>\*</sup> The nearest to proving it are Bornstein et alii (1951) who showed the principle present in pancreatic blood to be hyperglycaemic, glycogenolytic and independent of the pituitary-adrenal system."

In this connection it may be pointed out that the evidence that this substance is secreted into the blood stream rests on the cross-circulation experiments of Foa et alii (1949) in which the acinar part of the pancreas was still intact and on the not entirely conclusive results of

\* My underlining - *AC*.

Cavallero and Malandra (1950 b) in which again the acini are still functional. Lastly, the experiments of Bornstein, Reid and Young (1951) are of vital importance to the evidence behind the statement of de Duve (1953).



GENERAL INTRODUCTION TO PRESENT EXPERIMENTAL WORK.

It is hoped that the experiments which have been carried out in the work embodied in this thesis may throw additional light on the problem of demonstrating alpha cell secretion of glucagon.

The experimental work is in two parts.

(1) Attempts have been made to maintain "in vitro" alloxan diabetic dog pancreas, in which the ducts have been ligated 6 weeks or more. The supernatant fluid from these tissue cultures or 'organ cultures' has been extracted and assayed on intact rabbits in an endeavour to show whether a hyperglycaemic substance was secreted into the supernatant fluid in a way analogous to endocrine secretion. These studies have been correlated with histological observations on sections of the ligated alloxan diabetic pancreas and on sections of the explanted pancreas.

(2) Attempts have been made to prepare A.D.H.A. rats according to the method of Bornstein (1950) with the object of repeating the experiments reported by Bornstein, Reid and Young (1951) and adding to them by observing if growth hormone would still excite secretion of a hyperglycaemic substance into the portal vein after total pancreatectomy.

The first type of experiment postulates

an 'organ culture' of A-cell tissue, relatively 'pure' in a physiological sense, apart from fibroblasts, fat cells and a few B-cells, which might remain after alloxan administration. The possible effects of acinar tissue have been eliminated and the A-cell islets maintained 'in vitro' are functioning independent of any influence exerted by other endocrine organs.

It was felt the second type of experiment on rats should be attempted to obtain the additional information pertaining to A-cell secretion and its control by pituitary growth hormone that pancreatectomy would provide, and to confirm a work to which considerable weight has been attached (de Duve, 1953).

As each section in this thesis is self-contained, it is proposed that the relevant discussion and summary of results should take place at the end of the appropriate section, and consequently the general summary in section V will be brief. It is felt that only confusion would result from an attempt to review together all aspects of such widely differing series of experiments as for example the tissue culture ones in Section III and the A.D.H.A. rat ones in Section IV. Therefore, only these points relevant to the main aims of the thesis will be discussed in Section V.

During the assays performed in Section III it was found that human serum had an effect on the blood sugar of rabbits. A number of other proteins were then investigated to see if they had a similar effect. The results are not strictly relevant to this thesis, but as it was felt they might be of some interest they are recorded in Appendix iii.

The details of the clinical and pathological records of the various animals may be seen in an abridged form in Appendix ii.



SECTION II.

ACCOUNT OF THE PREPARATION OF ALLOXAN DIABETIC  
DOGS IN WHICH PANCREATIC LIGATION WAS PERFORMED IN  
ORDER TO SECURE ALLOXAN DIABETIC SCLEROSSED PANCREAS  
FOR TISSUE CULTURE.

Introduction

As described in Section III of this work, it was decided that to obtain a tissue culture of pancreatic alpha (A) cells in a state of relative physiological purity, it would be necessary to prepare an alloxan diabetic dog in which at least part of the pancreas had been ligated and allowed to sclerose, so that the B-cells of the islets would be largely destroyed and the exocrine tissue atrophied. The A-cells of the islets would be the only functional part remaining other than fat and connective tissue.

Methods and Materials

In the first instance it was decided to attempt to make the dog alloxan diabetic and subsequently operate on it.

The reasons for this particular order of events were: (1) It was considered initially that the administration of alloxan intravenously was the more uncertain part of the preparation and

that it would be a waste of time to operate first and then find the animal could not be made diabetic.

(2) Walpole and Innes (1946) had reported that alloxan diabetes could not be produced in rabbits if the pancreas had previously been ligated.

This had been confirmed in dogs by Adams (1949), although, admittedly, the time after ligation when he found alloxan ineffective was 75 days. This was an arbitrary time selected by Adams for his particular investigation, but it was thought that a less period might prevent the action of alloxan also. These findings were not in accord with La Barre and Hanquinet (1947) who had been able to produce alloxan diabetes one month after ligation of the pancreatic ducts of dogs. It was felt, however, that the risk of its being ineffective was not worth taking, as nothing was apparently to be gained by it.

The dose of alloxan was in the range recommended by Goldner and Gomori (1943) to produce diabetes without uraemia, i.e. 50-75 mgm/Kg. of body weight. Usually 50 mgm/Kg. were given. It was freshly prepared each time and given over a period of 2 minutes in 5% aqueous solution into the tibial vein. (Alloxan was supplied by Genatosan).

The animals were fasted for 18 hours before administration. Blood sugars were estimated before

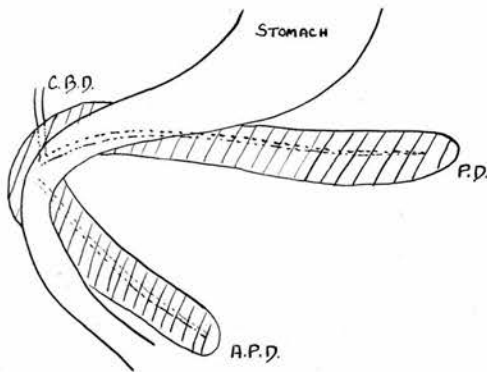
giving alloxan, again at approximately 4 - 6 hours after administration and then each morning (fasting) for several days. Blood was taken into a mixture of potassium oxalate and sodium fluoride and the sugar estimated by the method of Hagedorn and Jensen.

The animals were weighed at the beginning of each experiment and again from time to time subsequently.

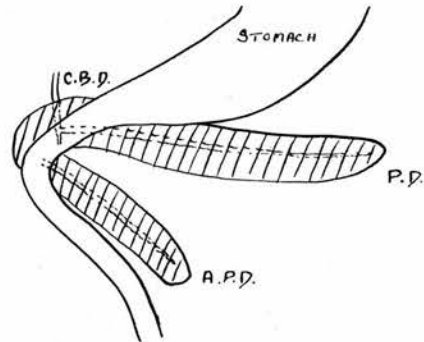
They had a normal unrestricted diet of horse meat, bread and mash with unlimited quantities of tap water. They were kept in dog cages. The temperature of the room was 18-19°C. A metabolism cage was not available and so urinary sugar estimations are infrequent, as urine could be obtained only when a dog passed it on being allowed out of its cage into the dog house for a short time.

Records were kept of all procedures and variations in the conditions of the animals and autopsy was performed on those that died. This information will be found in an abridged form in Appendix II a.

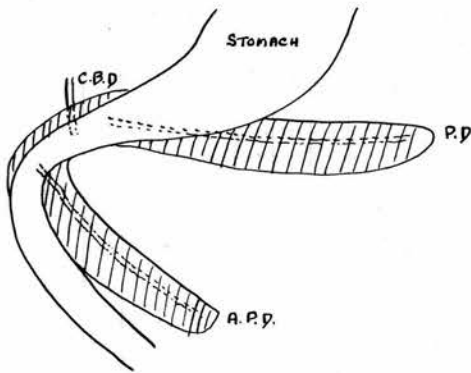
VARIATIONS IN ANATOMY OF PANCREATIC DUCTS AND COMMON BILE  
DUCT OF THE DOG (DRAWN AFTER BRADLEY 1948)



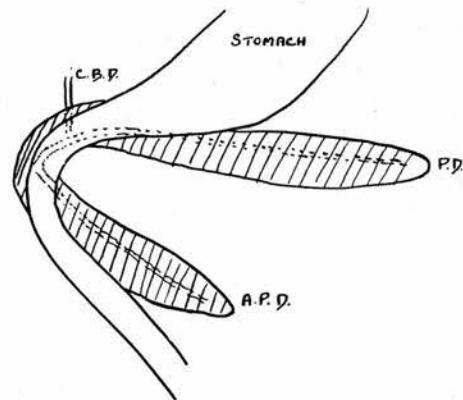
1. COMMON BILE DUCT AND PANCREATIC DUCT ENTER DUODENUM TOGETHER, ACCESSORY PANCREATIC DUCT ENTERS SEPARATELY. (MOST COMMON)



2. PANCREATIC DUCT JOINS COMMON BILE DUCT BEFORE THE LATTER ENTERS DUODENUM AND ACCESSORY PANCREATIC DUCT ENTERS SEPARATELY.



3. ALL THREE DUCTS ENTER SEPARATELY.



4. TWO PANCREATIC DUCTS ENTER TOGETHER, SEPARATELY FROM BILE DUCT. (VERY COMMON)

C.B.D. = COMMON BILE DUCT.  
 P.D. = PANCREATIC DUCT.  
 A.P.D. = ACCESSORY PANCREATIC DUCT.

Fig. 1.

Dog 1 was given alloxan by the method outlined above. The blood sugar showed the characteristic triphasic response seen after alloxan administration, and by the third day after injection the animal was clearly diabetic with a fasting blood sugar of 178 mgm.%; so it was decided to ligate one limb of the pancreas. The reasons for ligating one limb only as opposed to ligating the pancreatic ducts were as detailed below:

(1) Reference to fig. 1. showing the many possible arrangements of pancreatic ducts in the dog will indicate the difficulty of isolating each pancreatic duct and ligating it, as the ducts are deeply buried in the pancreatic tissue and much dissection is necessary to find them.

(2) It was considered that only as much ligated alloxan diabetic pancreas as was needed for tissue culture need be prepared.

(3) The dog would undoubtedly survive in better condition if some exocrine tissue remained to supply pancreatic ferments to the gut.

Accordingly, a type of operation was employed which was a modification of the technique used by MacCallum (1909) (fig. 2).

The dog was given 0.3 G. sodium thiopentone intravenously. An endotracheal tube was then passed and connected to an open circuit anaesthetic apparatus charged with ether.



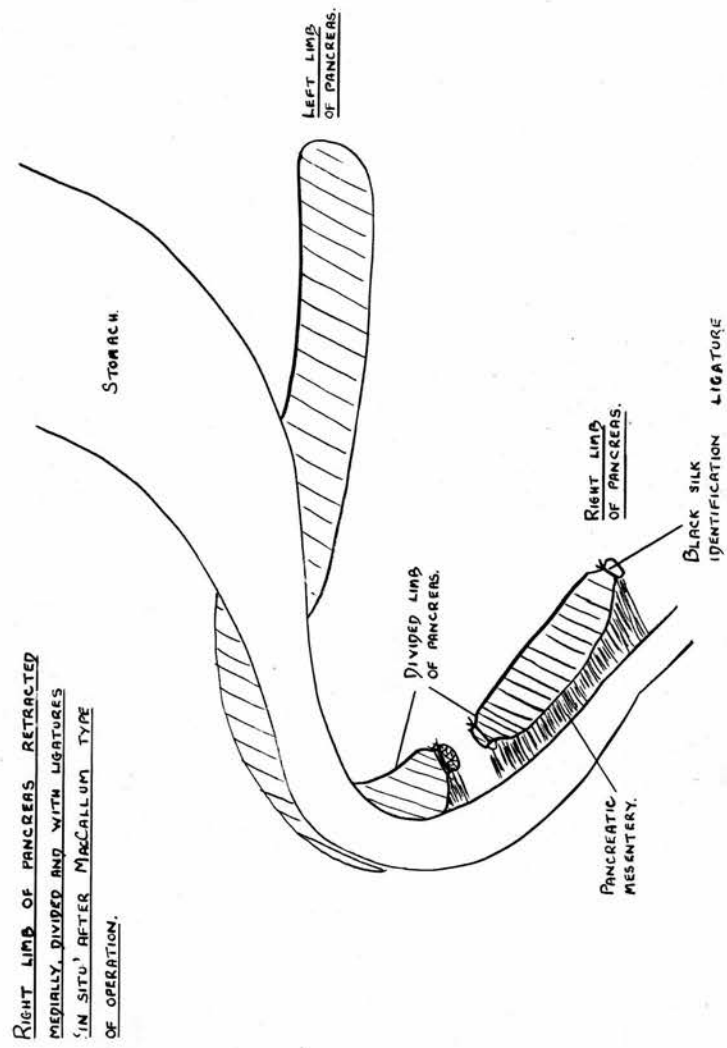
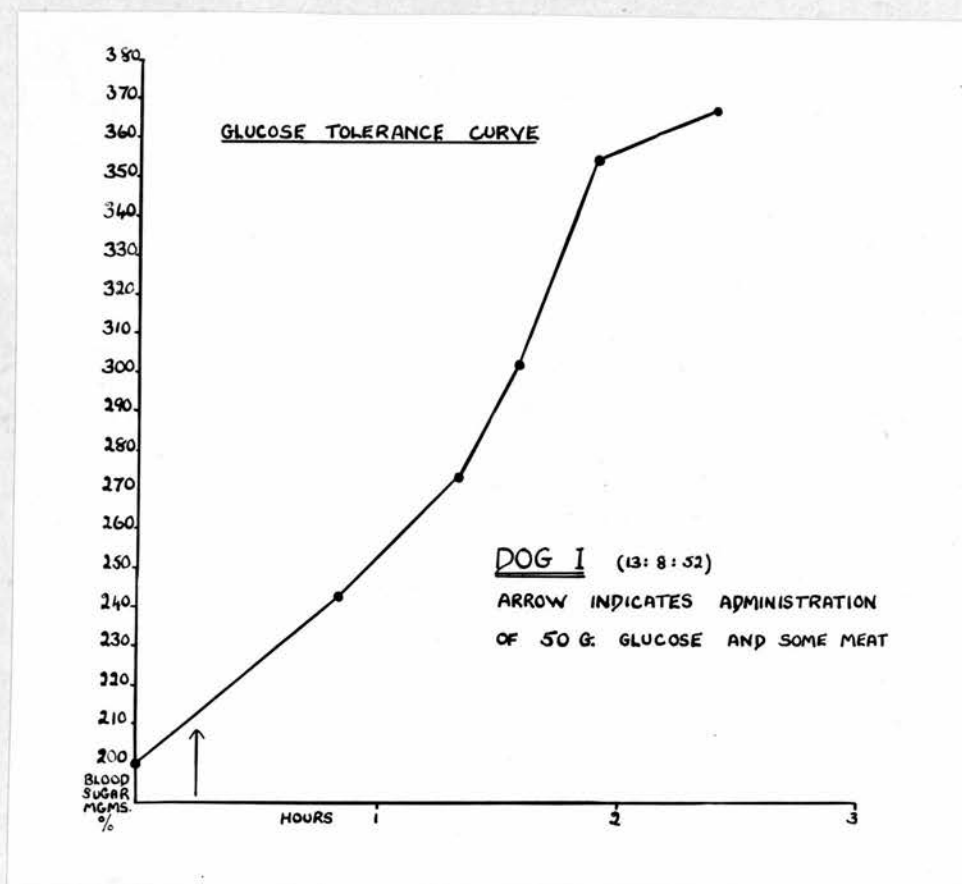


FIG. 2.

An upper right paramedian abdominal incision was made passing through skin and muscle. The peritoneum was opened and peritoneal towels attached. The right limb of the pancreas was readily exposed as it lay in its mesentery behind and internal to the duodenum. A small hole was cut in the pancreatic mesentery and 3 black silk ligatures were tied around the whole right limb at a point 1 c.m. distal to the head of the pancreas. The pancreas was divided between the most caudal ligature and the other two, thus leaving 2 ligatures to close the severed proximal portion. Both cut ends were then coagulated with the electric cautery. A piece of great omentum was laid over the cut ends. A black silk ligature was placed at the most distal part of the right limb to facilitate subsequent identification of the sclerosed limb.

The abdomen was closed in layers. Streptomycin powder was sprinkled on the peritoneum and sulphonamide - penicillin powder on to the divided muscles and subcutaneous tissue. In a few days the animal was quite recovered from the operation.

A specimen of urine obtained reduced Benedict's solution to an orange colour. A glucose tolerance test was done by giving 50 G. glucose mixed with horse meat. The result seen on graph 1. shows a diabetic type of curve.



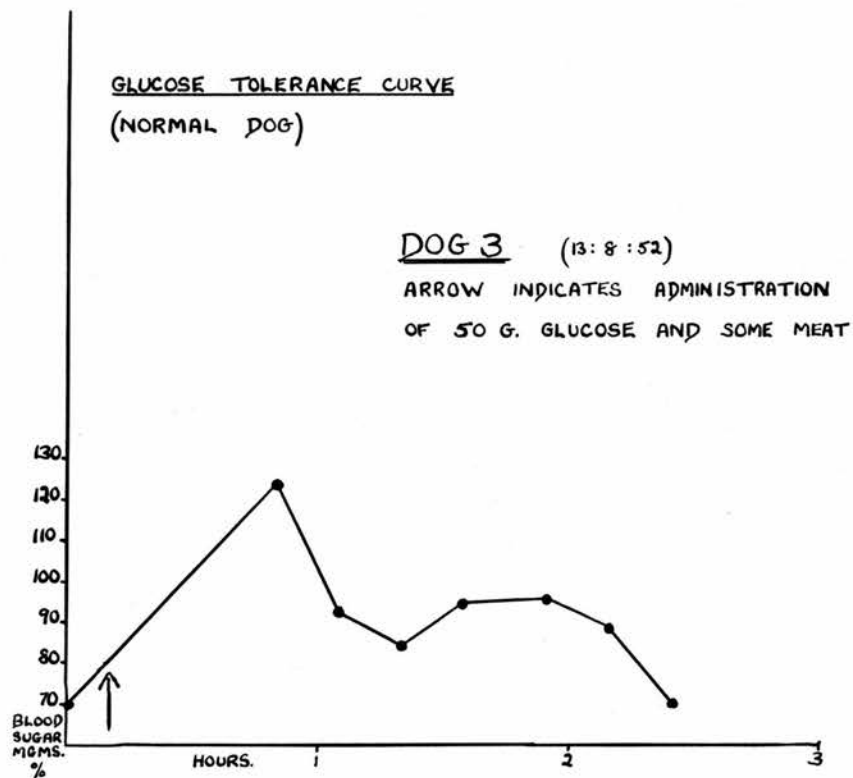
Graph 1.

At the same time a glucose tolerance test was done on a normal dog (Dog 3.) for comparison. (c.f. graph 2.)

It was decided that the animals being prepared for the tissue culture experiments should not be maintained on insulin preparations (a) lest such preparations, containing a glyco:genolytic factor, might affect the dog's alpha cell function. Also (b) very severely diabetic animals would be more difficult to maintain.

Nevertheless, it was felt there was no reason why an animal should not have insulin to tide it over a short term illness. This was done with Dog 1. which became a little drowsy and vomited on one occasion. At this time the animal had 5 units of globin insulin and 50,000 units of penicillin daily for three days, but no insulin at any other time.

Dog 1 was sacrificed approximately 5 months after the pancreas was ligated. 0.5 G. sodium thiopentone was given rapidly to kill the dog. The abdomen was quickly opened and the sclerosed limb of pancreas identified by the black silk ligatures. It was removed aseptically and placed in Tyrode solution in a boiling tube. The ligated diabetic pancreatic tissue was then taken at once to the tissue culture room where everything had been made ready for culturing. The cultures were



Graph 2.



set up in the way described in Section III, Experiment 1. of this thesis. A specimen of urine obtained at post-mortem produced a brick-red precipitate with Benedict's test for sugar and was strongly positive with Rothera's test for acetone. (Autopsy findings are recorded in Appendix ii a.)

As it was expected that further tissue culture experiments would be necessary, attempts were made to prepare another animal similar to Dog 1., but considerable difficulty was encountered.

Dog 3 was given 60 mgm. alloxan/Kg. of body weight, but died 48 hours later. At post mortem and subsequent microscopy the pancreatic islets were found much reduced in number and size, and the renal tubules were damaged. The liver showed fatty vacuolation. It was considered death had resulted from diabetes and uraemia.

Dog 4 was given 60 mgm. of alloxan/Kg. of body weight. Hyperglycaemia was present the following morning. A permanent diabetes of moderate extent ensued (fasting blood sugar 183 - 292 mgm.%.). At this time it was contemplated repeating the work of Roderiguez-Candela (1947) in estimating the insulin requirements of dogs before and after ligation of the pancreatic ducts. (This line of work was, in fact, not continued). As this would require a complete duct ligation and not the partial limb ligation done on Dog 1. the



opportunity was taken to gain experience of this type of operation. It was found to require considerable dissection to determine which particular duct arrangement this dog had. As a result the blood supply to part of the duodenum was impaired. The dog died the following day of shock, and gangrene of the duodenum.

Dog 5 was given 60 mgm. of alloxan/Kg. of body weight. There was no triphasic response of the blood sugar. The fasting blood sugar was 89 mgm.%. before alloxan and 96 mgm.% after alloxan, and the urinary sugar was negative. Alloxan was given again, but diabetes did not result. As this dog had failed to become diabetic it was discarded.

Dog 6 was given 60 mgm. alloxan/Kg. of body weight. It became quickly and very severely diabetic and within 5 days had a fasting blood sugar of 700 mgm.%. It died on the 6th day after receiving the alloxan. Autopsy showed severe renal tubular lesions, foaminess of the liver and fatty degeneration, and severe damage to the pancreatic islets. It was considered that death resulted from uraemia and diabetes, and that the very high blood sugar to some extent was due to an inability to excrete the excess sugar.

As the operation of ligating the pancreatic ducts themselves as opposed to ligating the limb

of the organ seemed more hazardous, it was decided to operate on Dog 7 first and give alloxan 7 days later. A complete ligation of all pancreatic ducts was performed. The prolonged operation time and extensive dissection necessary for this procedure produced shock and damage to the duodenum and pancreas, from which the animal died within 24 hours.

Dog 8 was given 0.3 G. sodium thiopentone which caused it to collapse and die during induction of anaesthesia.

Dog 9 had the pancreatic ducts successfully ligated. Alloxan was given in a dosage of 50 mgm/Kg. of body weight 6 days after operation, but failed to make the animal diabetic. Alloxan was again given 20 days after operation in a dose of 60 mgm/Kg. of body weight, but the animal did not become diabetic even after the second dose.

Since it was now thought that operative technique was improving and alloxan was so uncertain in its action, some dogs dying and others not becoming diabetic, a reversion was made to the former procedure of giving alloxan first.

Dog 10 had 60 mgm./Kg. of body weight and died within a week with a raised blood urea and a raised blood sugar, and was considered at autopsy to have died of diabetic-uraemic syndrome.

Dog 11 was given 60 mgm. alloxan/Kg. of body weight, but failed to become diabetic.

Dog 12 had 60 mgm. alloxan/Kg. of body weight and died in 4 days with a high blood sugar. At autopsy there was renal damage and a little bronchopneumonia and pulmonary collapse. Marked centri-lobular fatty degeneration of the liver was found. This animal was considered to have died of diabetes and uraemia.

Dog 13 was given 50 mgm. alloxan/Kg. of body weight and responded by becoming mildly diabetic. It remained in this condition for some weeks during which an operation to ligate the right limb of the pancreas was performed. Duct ligation was not done as the project to estimate insulin requirement before and after ligation of the ducts had been abandoned meantime. Finally, after a glucose tolerance test (c.f. Appendix ii a.) and many fasting blood sugars and urinary sugars, it became evident that the animal was only mildly diabetic and that the diabetic state was not worsening. Alloxan was given again, but the diabetes was unaffected, so this animal was discarded.

Dog 14 was given 60 mgm. alloxan/Kg. of body weight and died within 8 days of diabetes and showing renal damage.

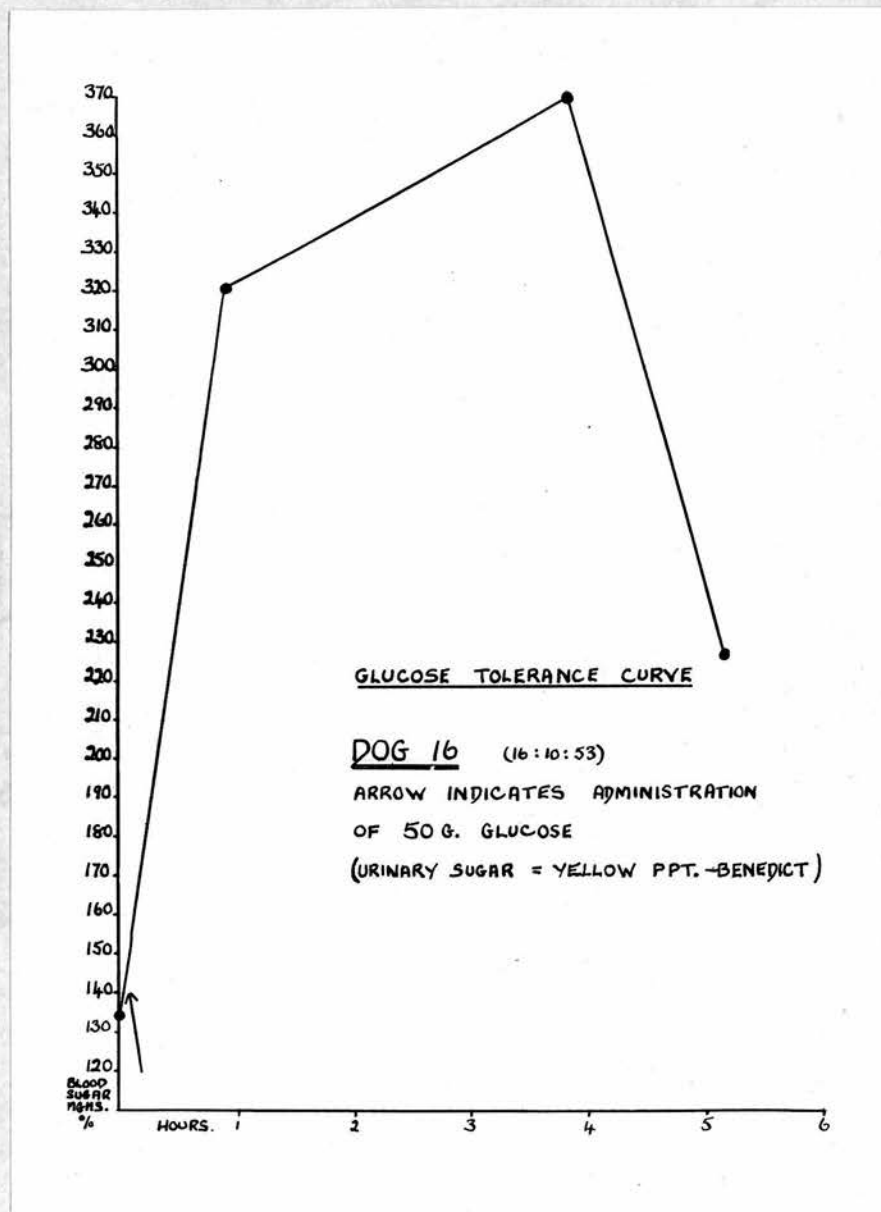
DITHIZONE AND OXINE DIABETES.

Since it was proving so difficult to prepare an alloxan diabetic animal with a ligated pancreas the possibility of making the dogs diabetic with dithizone or oxine was considered. These substances had been reported by Kadota (1950) as producing diabetes in rabbits. Oxine could not be obtained, but a supply of dithizone was finally provided by Messrs. Hopkins and Williams Ltd. Kadota (1950) recommended a dose of 50-200 mgm. of dithizone/Kg. of body weight dissolved in 0.2 - 0.5% ammoniacal solution and given intravenously.

In the present work it was attempted to prepare an injection of 75 mgm./Kg. of body weight in the first instance. Kadota's instructions were followed exactly, but this amount could not be dissolved even at a dilution of 0.5%. The solubility of dithizone was proved to be of the order of 0.1% in ammoniacal solution. This was also found by Root and Chen (1952). In a 10 Kg. dog to give 75 mgm. dithizone/Kg. would entail an intravenous injection of 750 c.c. of the saturated solution, which is clearly impracticable. It was attempted to dissolve this substance in stronger alkaline solutions, in acid solution, in chloroform, and by warming, without any significant improvement in the amount dissolved. Enquiry was made and it appeared that the procedure adopted by Kadota was to attempt to

dissolve the appropriate dose in 20 c.c. of ammoniacal solution after warming to 60 - 70°C. for 10 minutes - then to filter off, leaving the undissolved residue and injecting the filtrate (Kadota, 1953). This being so, the matter was not investigated further.





Graph 3.



To overcome the difficulties encountered with alloxan a different plan was adopted. This was to operate on the animal first and wait 6 weeks until the exocrine pancreas had atrophied and then to give alloxan. If the animal was clearly going to die of diabetic-uraemic syndrome, it would be killed and the ligated half of the pancreas thus obtained would be diabetic and suitable for immediate culturing. Such a procedure had not been adopted before, because of doubt as to whether a ligated pancreas would respond to alloxan 6 weeks after ligation (Adams, 1949). However, during the course of the present work it was found that Gaede et alii (1950) had been able to produce diabetes in puppies 3 months after pancreatic duct ligation, so that at 6 weeks after ligation there might be no difficulty.

This was carried out on Dog 16. The right limb of the pancreas was ligated in the same way as was done with Dog 1, black silk markers being left at either end to ensure subsequent identification. The dog did well after the operation, and 43 days later alloxan was given in a dose of 60 mgm./Kg. of body weight. A good triphasic response was obtained and the fasting blood sugar was raised from 69 mgm.% before alloxan to 132-165 mgm.% afterwards. The glucose tolerance test showed a clearly diabetic type of curve (c.f. graph 3), and the urinary sugar reduced Benedict's

solution to a yellow colour. No acetone was detected, and the dog did not develop a severe diabetic-uraemic state.

When diabetes was certainly known to be present the animal was anaesthetized in the usual way with sodium thiopentone and ether, using endotracheal intubation. The abdomen was opened with surgical asepsis, and the atrophied right limb removed and put in sterile Tyrode solution for the tissue culture experiments described in Section III, Experiment 2. A further 0.5 G. sodium thiopentone was given into a mesenteric vein to kill the dog.

The ligated diabetic pancreas in Tyrode was taken at once to the tissue culture room which had been made ready.

(For autopsy findings and case history c.f. Appendix ii a.).

(Dogs 2 and 15 were used in other experiments not included in this thesis.)

Alloxan diabetic sclerosed pancreas successfully obtained.	Died of diabetic-uraemic syndrome	Died as a result of operation	Died of anaesthesia	Failed to become diabetic
Dogs 1, 16.	Dogs 3, 6, 10, 12, 14.	Dogs 4, 7.	Dog 8.	Dogs 5, 9, 11, 13.

**TABLE A.** Analysis of the preparation of alloxan diabetic dogs with sclerosed pancreas.

Total number of dogs given alloxan	12
Number of dogs which died of diabetic-uraemic syndrome due to alloxan	5
Number of dogs which failed to become diabetic after alloxan	4
Number of dogs which became successfully diabetic and lived after alloxan	3

**TABLE B.** Shows the effect of doses of 50 - 60 mgm. alloxan/Kg. of body weight on 12 dogs.

Response to alloxan	Numbers of Dogs. (+) after the number indicates a triphasic response of blood sugar.	Proportion showing triphasic blood sugar response.
Became diabetic and lived, or died of diabetic-uraemic syndrome	1(+), 3(+), 4(-), 6(+), 10(-), 12(+), 14(?+), 16(+).	$\frac{5-6}{8}$ 60-70%
Failed to become diabetic	5(-), 9(-), 11(-), 13(-).	$\frac{0}{4}$ 0%

**TABLE C.** Incidence of triphasic glycaemic response in relation to the ultimate effect of alloxan.

### Discussion

It is evident from the results shown on Tables A and B that a moderate degree of diabetes is not readily produced in dogs with alloxan, only 3 out of 12 (25%) animals surviving, diabetic, in this series. These are very low figures compared with some other species, e.g. the rat where generally approximately 50% can be made diabetic (Gaarenstroom, 1946) - (This thesis, Section IV). It is also less than one might expect from the experience of Goldner and Gomori (1943) who, giving doses of 50 - 75 mgm./Kg. of body weight to 6 dogs, found 4 were permanently and severely diabetic. These authors state that a dose of 50 - 75 mgm./Kg. of body weight should produce diabetes without renal complications, that a dose of 75 - 100 mgm./Kg. of body weight produces a diabetic-uraemic syndrome with severe damage to the renal tubules, and that a dose of over 100 mgm./Kg. of body weight causes death in a few hours.

It is seen from Table B. that nearly half of the animals developed diabetic-uraemic syndrome and died with the minimal dose recommended by Goldner and Gomori (1943), which should have produced only typical diabetes without renal lesions. The findings in the present work are more in accord with those of Thorogood and

Zimmerman (1945), who gave 50 - 65 mgm./Kg. of body weight to 43 dogs. They found 7 developed glycosuria (severe), and 13 developed uraemia, and the remainder showed only transient glycosuria and were discarded. They wonder if the difference in response was a manifestation of individual variation or was due to differences in the samples of alloxan.

In the present work the same alloxan was used all the way through the work, and so it seems probable that it is a matter of individual variation.

When alloxan is injected the blood sugar often rises after a few hours to fall to a lower level some 12 - 18 hours later, causing hypoglycaemic convulsions in some species such as rabbits, though not usually falling so low in the dog. By 36 - 48 hours after injection permanent hyperglycaemia occurs if the injection has been successful. This triphasic response is thus composed of (1) hyperglycaemia of a temporary nature followed by (2) hypoglycaemia and then (3) a permanent hyperglycaemia. Reference to Table C. shows that in the animals which became successfully diabetic or died of alloxan, the triphasic response was usually observed. In animals which failed to become diabetic or became very mildly diabetic, there was no triphasic response. It is of some interest, therefore, to determine the blood sugar



4 - 6 hours after giving alloxan. If it is raised the animal will probably become diabetic. If it is not raised the animal is unlikely to become diabetic.

#### Summary

- (1) 14 dogs were used in an endeavour to secure alloxan diabetic sclerosed pancreas for tissue culture.
- (2) This tissue was successfully obtained on 2 occasions.
- (3) It was found that only a relatively small number of the dogs could be made successfully diabetic, and that a larger number failed to become diabetic or died as a result of a diabetic-uraemic syndrome.
- (4) It was noted that the dose of alloxan necessary to produce diabetic-uraemic syndrome was smaller than that indicated by Goldner and Gomori (1943).
- (5) These differences are thought to be due to individual variation in the animals.
- (6) The value of the triphasic response of the blood sugar as a prognostic sign after alloxan administration is discussed.



SECTION III.

AN ACCOUNT OF TISSUE CULTURE EXPERIMENTS WITH  
SCLEROSSED ALLOXAN DIABETIC DOG PANCREAS AND OF  
THE BIOLOGICAL ASSAYS OF THE SUPERNATANT FLUIDS  
OF THE CULTURES.

Introduction and Preliminary Work.

As outlined in the General Introduction to this thesis it was proposed to set up an "in vitro" system where alpha cells (A-cells) might continue to function in a state of relative physiological 'purity' and might be shown to secrete a hyperglycaemic factor in a way analogous to endocrine secretion, whilst removed from the influence of the other endocrine organs.

In the first place it was thought an attempt should be made to get a pure culture of A-cells, removing whole islets from the pancreas by micro-dissection. If such a technique could be developed, then it would be a relatively easy matter to obtain a pancreas containing A-cell islets from an alloxan diabetic animal.

Pancreas was removed from freshly killed rats and sections cut  $30\mu$  thick on a freezing microtome. These sections were placed on a slide and covered with physiological saline. After some practice it was found possible to identify islets in an unstained specimen.

A variety of cutting tools was used in the micromanipulator including dissecting needles, finely drawn glass rods and fine, straight cutting needles.

It was discovered, however, that the islets are firmly adherent to the pancreas and cannot be enucleated even when the section is treated with hyaluronidase to loosen intercellular substance. On one occasion, using hyaluronidase and straight cutting needles, an islet was cut out by sawing tangentially round it. This procedure took over 2 hours.

However, the trauma of freezing and sawing for such a prolonged time made it unlikely the cells would have survived. In addition some exocrine tissue was probably removed as well.

This technique was not considered practical and was therefore abandoned.

It was considered the same end might be achieved in a more efficient way by preparing an alloxan diabetic animal and ligating the pancreatic ducts, allowing the exocrine tissue to sclerose and culturing the alloxan diabetic sclerosed pancreas. The only functional tissue remaining would be the A-cells, possibly a very few B-cells surviving the alloxan, fat and connective tissue.

The dog was considered the most suitable animal for this purpose because:

- (1) Its pancreas more closely resembles that of man than does the pancreas of rodents.
- (2) It would be possible to ligate half the pancreas in a dog and possibly to ligate all ducts if necessary.
- (3) A complete pancreatectomy could be performed if the development of the experimental work should demand it. In rodents due to the feathery nature of the pancreas 100% pancreatectomy would be uncertain (rabbits, rats).
- (4) It had been stated by Duff (1945) and by Goldner and Gomori (1943) that a wide margin existed between the diabetogenic and the uraemic or lethal dose of alloxan in the dog. As seen in Section II of the present work, this did not turn out to be the case.

TISSUE CULTURE - A BIOLOGICAL RESEARCH TECHNIQUE.

Tissue culture is not a new method of biological research. It was used to solve a particular problem of chick development by Roux in 1885. The technique of explantation was developed and enlarged by Harrison, who laid the basic foundations of the methods used today. The art of culture was greatly extended by Alexis Carrel, who introduced many new media and ways of explantation. Many other workers have contributed methods of tissue culture and new techniques amongst them Maximow and Strangeways, and more recently Fell.

Tissue culture techniques have been applied to a variety of biological research projects. Tumour and embryological problems have been particularly studied in this way. Almost all tissues, however, have been successfully explanted and maintained alive or grown 'in vitro'. Amongst them have been complex organs maintained in a functional state. Thus by suppressing cell multiplication, iris epithelium has been found to make pigment (Doljanski, 1930) and thyroid to form colloid (Ebeling, 1925). Pancreas has been successfully cultured 'in vitro' for a period of one month (Kapel, 1926), although in this case no particular study of its functional activity was made.



The literature on tissue culture is enormous, and since its technical aspects appeared to be in many ways a skill which could be learnt very much more readily by seeing and doing than by reading, it was thought necessary to spend some time in a tissue culture laboratory to learn the techniques and principles of the subject. In fulfilment of such a wish I was privileged to work for a little in The Strangeways Research Laboratory, Cambridge under the direction of Miss Honor B. Fell. As tissue culture is largely an empirical art dependant entirely on suitable technique, it is proposed to discuss in some detail the methods from which the particular techniques used in this work were derived.



1. The Strangeways method of tissue culture.

In general the approach to tissue culture recommended by Fell is characterised by simplicity and the practical yardstick of "Does it work?"

Each worker when culturing does so in a private room, and visitors do not enter whilst culture is in progress. Some of the rooms have an ante-room where sterile glassware is kept, and where a technician works, either culturing or preparing media.

The rooms themselves are standard, small, biological laboratories with adequate bench space, but no special floor coverings and no elaborate colour schemes. The apparatus is sterilized by dry heat or in the autoclave in a sterilizing room, which serves all rooms in the laboratory. The rooms have windows. Chick plasma is obtained by bleeding cockerels on the premises. Tyrode and other saline solutions are also made up at Strangeways.

The apparatus used illustrates the emphasis on simplicity and improvisation. Instruments are kept and sterilized in old metal cigarette tins. During culture when the instruments are not in use, the working ends are placed under the shelter of the lid

of the tin and the handles allowed to protrude. Pipettes during an experiment when not in use have their working surfaces protected from aerial bacterial contamination by an open ended glass specimen jar laid flat.

The worker does not wear elaborate gown and head covering, only a white coat. No masks are worn. It has been found sufficient to prevent droplet contamination that no one in the culture room should speak when a culture is exposed, and that unnecessary moving about or opening of doors should be eliminated. The worker is not scrubbed up beforehand, the hands are simply washed and then wiped with cotton wool soaked in spirit. For 30 minutes before culturing, and all the time culturing is in progress, a beaker of distilled water is kept boiling in the room

- (1) to lay the dust and prevent dust-borne contamination;
- (2) to quickly wash out and sterilize pipettes.

The method of working is a modified 'no touch' technique of asepsis. A culture is never left uncovered for more than is absolutely necessary to perform the desired operation, and as far as possible it is protected from dust or droplet infection by a petri-dish lid held over it. No working

surface of any instrument or piece of glass-ware is ever touched by hand. Using these methods contaminated cultures have been almost unknown at Strangeways for many years.

## 2. More elaborate methods of tissue culture.

Whilst performing the preliminary experiments in this work I had the opportunity of visiting Dr. Albert Fischer's tissue culture laboratory at Carlsberg biologiske fondet in Copenhagen.

This institute was originally designed and built for tissue culture work. The walls are of granolithic material and are faired into the floor to eliminate dust collection. The floors are of polished black rubber. The workers are dressed entirely in black. Black gowns, hoods and face masks are worn in the culture rooms. The working bench is covered with black sateen. The purpose of the prevailing dark colouring is to render the light coloured explants more readily visible. The workers scrub up before culturing, as for a surgical operation. Talking is permitted whilst working and people may move about. The preparation rooms and sterilizing rooms are very large and resemble those of an operating theatre. The opportunity was given me to assist at a number of culturing sessions and there was no doubt that the general atmosphere and colouring was pleasant to

work in.

A laboratory of this type was obviously enormously expensive and was clearly designed for workers who were engaged full time on problems of tissue culture technique itself, rather than in applying the techniques to isolated problems of biological research over relatively short times, as the tendency was at Strangeways. This system of working is similar to the one described by Parker(1950) as in use in his laboratory, having been modelled on the laboratory of Alexis Carrel at the Rockefeller Institute for Medical Research in New York.

It was evident that in setting up a small tissue culture laboratory in Edinburgh with the intention of using a tissue culture technique for solving a particular problem that the Strangeways method with its practical approach and accent on improvisation was the one of choice, as a laboratory adequate to attempt the problem in hand could be equipped at relatively small cost.

DESCRIPTION OF THE LABORATORY AND METHODS.

A spare dark room was made available. It was air conditioned and windowless. A sheet of glass 18" square on the bench was used as a working surface as it could be readily cleaned with spirit.

Cleaning and Sterilizing.

Metal instruments used in preparing embryo extract and cutting explants, etc. were sterilized in cigarette tins as at Strangeways. Glass-ware was bought new and was prepared in the following way to get rid of the alkalinity in new glass which can inhibit tissue cultures.

- (1) Glass left in 1% H.Cl. for several hours.
- (2) Rinsed in tap water.
- (3) Boiled for 10 minutes in water and soap jelly.
- (4) Rinsed in warm tap water.
- (5) Boiled again in soap jelly as before.
- (6) Rinsed in warm running water.
- (7) Left in distilled water 30 minutes.
- (8) Rinsed in 95% alcohol.
- (9) Dried in oven.

Used glass-ware was treated the same way except (1) was omitted (Parker, 1950). Glass was wrapped in kraft paper and sterilized by dry heat, placing it in the hot air oven for 90 minutes at 160°C. All glass vessels such as Carrel flasks,

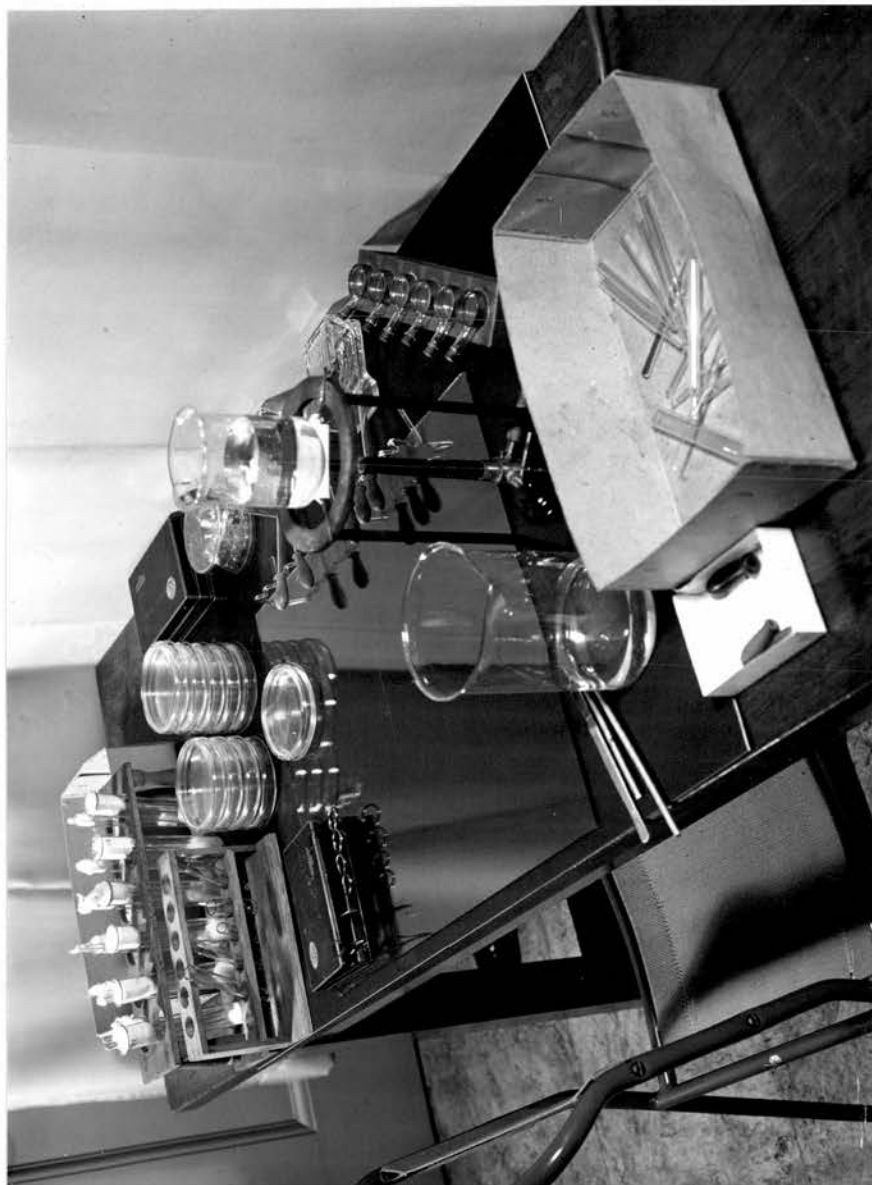


Fig. 3.

Working arrangements for culturing, using Carrel flasks. The sterile glassware, instruments and reagents are to the left of the worker and the used glassware to the right.



test-tubes, etc., having narrow openings, had them loosely stoppered with cotton wool.

Metal instruments and rubber stoppers, etc. were sterilized in the autoclave as dry heat damaged them. They were given 30 minutes at 17 lbs. pressure. All glass-ware and sterile apparatus were sterilized the day before use and kept in their wrappings or boxes in a closed metal cupboard.

#### Reagents.

Embryos for making the embryo extract were obtained by collecting unincubated fertile hen eggs and setting them up in an egg incubator. The embryos were used 8 - 11 days after incubating, but generally after 10 days.

Cockerel plasma and Tyrode solution were obtained from the Wilkie Surgical Research Institute and stored at  $-25^{\circ}\text{C}$ . All other sera and physiological solutions used in this work were stored at this temperature.

#### Working layout (fig. 3.)

When working, all clean unused glass-ware was kept on the left and all contaminated, discarded glass-ware was passed to the right side. Pipettes actually in use were kept in specimen jars as described above with the teat-ends supported

on a small rack. These, for convenience, were situated in front of the worker. It was found easier to see the cultures when working with an 'Anglepoise' lamp as the sole means of illumination. Cultures were examined with a binocular dissecting microscope using transmitted light. Direct illumination of the cultures did not display the darkening which indicates cultures are unhealthy.

SUITABLE TECHNIQUES FOR PROPOSED EXPERIMENT.(1) Growth and function.

The alloxan diabetic, sclerosed pancreas was to be explanted with a view not to promoting growth, but to maintaining differentiation of the A-cells and encouraging normal function as far as possible. If growth is allowed, then, correspondingly, specialised function declines as the cells de-differentiate. It has been found in tissue culture work that the amount of embryo extract used in the medium determines chiefly whether the culture will grow or remain static. In most types of experiment, growth is desirable, because without growth the explants will soon die. However, where physiological function is necessary, the amount of embryo extract is cut to a minimum, only enough being used to clot the plasma. Such an explant, of course, has a relatively shorter life than a growing culture, but that is not important as enough time should be available to carry out the desired experiments.

(2) Culture technique employed.

Clearly, if fluid is to wash the cultures which are to secrete into it, a technique must be employed using either a fluid medium or a solid medium with a fluid phase. Parker (1937) used

a fluid medium in Carrel flasks for culturing washed rabbit spleen which had been immunized against sheep red cells. He succeeded in demonstrating in this fluid a haemolytic antibody. The cultures were maintained only 4 days. (This experiment has, of course, nothing to do with the proposed pancreatic explantation, but it illustrates a successful physiological experiment in tissue culture). This is the disadvantage of the entirely fluid medium technique, that while suitable for short term functional experiments, survival of the explants is very brief. It amounts rather more to "organ culture" than to tissue culture, for no growth occurs.

A more stable and yet potentially functional type of experiment can be performed using Carrel flasks with the explant embedded in a permeable plasma coagulum and the latter bathed in a serum-Tyrode supernatant fluid. By not transferring the cultures from the flasks, growth can be further inhibited.

Using this technique, breast muscle of a chick has been kept alive for 12 months in the same flask (Parker, 1936) and cultures of a mouse mammary carcinoma have been kept alive for 6 months without transplantation (Parker, 1950).

It was decided, therefore, that the biphasic technique in a Carrel flask would probably be the

most suitable method. On this basis experimental work was begun.

### PRELIMINARY TISSUE CULTURE EXPERIMENTS.

In order to gain some experience of the techniques learnt at Strangeways and performed under supervision, it was thought essential to practise standard procedures such as the preparation of embryo extract and successful growth of fibroblastic cultures, before attempting the proposed experiments with diabetic pancreas.

In the first place coverslip cultures, being the simplest, were made until the results were reasonably good. Embryo extract was prepared in the following way which was the method used throughout the tissue culture work.

#### Preparation of Embryo Extract.

An embryo, preferably 10 days incubated, was taken directly from the incubator to the culture room to avoid cooling which might kill it.

The tissue culture equipment is set out in the way described and illustrated (fig.3). The egg is cleaned with spirit as are the hands of the operator, and is placed in an egg cup. The top of the egg is chipped around with the sterilized handle of a Bard-Parker knife. The top is removed in one piece by sterile forceps. The membranes are cut away with fine curved scissors and the embryo is then seen. It is lifted out



by the neck, using bent tipped fine forceps to avoid decapitating it, and is then laid in a watch glass in a petri dish. It is washed free of blood with Tyrode. The eyes and gall-bladder are removed, and it is minced very fine with the curved scissors for at least 5 minutes. After diluting 1:4 with Tyrode solution a very wide tipped Pasteur pipette is used to transfer the rather porridge-like product into a small hard glass tube measuring  $2\frac{1}{2}$ " x 1". The extract is now ground with a glass rod "until the operator is exhausted." (H.B. Fell.) After this has been achieved the tube is centrifuged at 3000 rev/minute for 20 minutes. The resulting supernate is a milky, rather opalescent fluid which should be quite free from cells. It is removed to another tube and now constitutes the embryo extract. It was made up freshly each day that it was required. (In the practice work, the heart was removed before mincing, and covered with Tyrode. So long as it was at 37°C. it would continue to beat. The heart was cut up later and explanted.)

The method of coverslip culture will not be detailed here as it was not used in the pancreatic explantations.

#### Preliminary Carrel flask cultures.

After proficiency had been attained with

coverslip cultures, explantation was started into Carrel flasks of diameter 3.5 cm. (D.3.5 flasks).

This work was undertaken with the object

- (1) of obtaining experience of using these flasks;
- (2) of determining suitable volumes of reagents required to embed the explants and to ensure the clot was adequately flooded by supernatant fluid;
- (3) of determining the size of explant which could be cultured successfully.

Clearly in the pancreatic explantation experiments it would be desirable to have as large a volume of tissue as could be nourished and oxygenated. It was necessary to determine these facts on heart cultures, because the pancreatic explants would be available only for the actual experiments. After one or two attempts it was found possible to keep 5 out of 6 chick heart explants alive and healthy for over a month without changing the flasks.

CONCLUSIONS FROM PRELIMINARY EXPERIMENTS.

(1) It was found a suitable size for explants was 1 - 1.5 m.m. in all diameters. If they were bigger than that they could not readily be maintained alive.

(2) It was also noted in these experiments that one of the most important factors in getting a healthy explant was to ensure that it was cleanly cut with a very sharp instrument. If there was any tearing or crushing, the explant tended not to survive. In the first instance cataract knives were used as the standard method of cutting up tissue for explantation, but these were found to bend easily at the junction of blade and handle, and were very expensive to replace. A supply of Borrowdale dissecting needles was obtained, and it was found a clean cut could be made by using one in each hand and drawing them close together in opposite directions through the tissue. This gave excellent results and was used in the first pancreatic explantations, although by the second attempt, it was found an even better result could be got by using two new single edged 'Eveready' razor blades in the same way.

(3) The clot that was found most suitable was 1.5 m.m. thick and consisted of 25 drops (1 c.c.) of 2 parts cockerel plasma and 1 part of Tyrode. To this was added in the flask 1 drop of 1:4

embryo extract which was found as rule sufficient to secure a firm clot with fresh plasma.

(4) The clot was patched in 24 hours with 0.5 c.c. (approximately) of 2:1 plasma Tyrode mixture and 1 drop of 1:4 embryo extract added.

(5) In 48 hours the clot and explant were flooded with a supernate consisting of Tyrode 0.1 c.c. and human serum 0.5 c.c.

(6) Changing the serum-Tyrode mixture every 3 days was found to be adequate.

(7) It was found that for transferring explants into the flask from the petri dish where they were cut, that a medium sized Pasteur pipette was better than a platinum loop as was used originally.

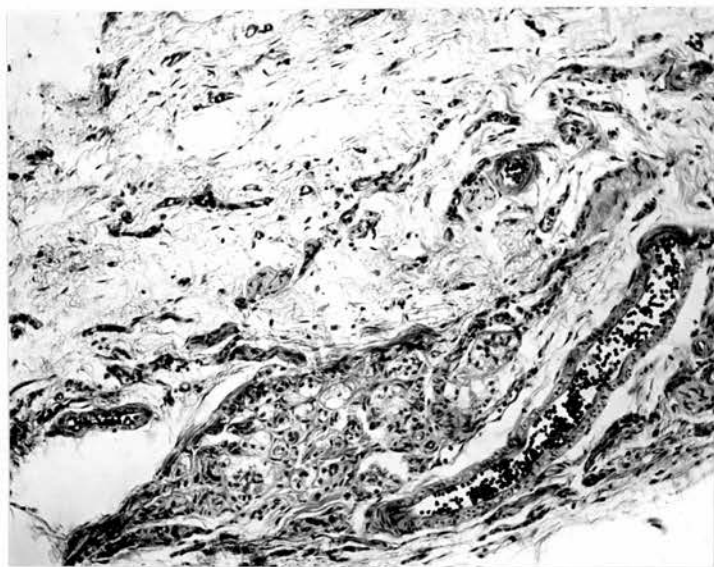


Fig. 4.

Dog 1. Sclerosed diabetic pancreas.  
Haematoxylin and Eosin. x 90.  
Atrophic acini surrounded by fibrosis  
and fatty infiltration.

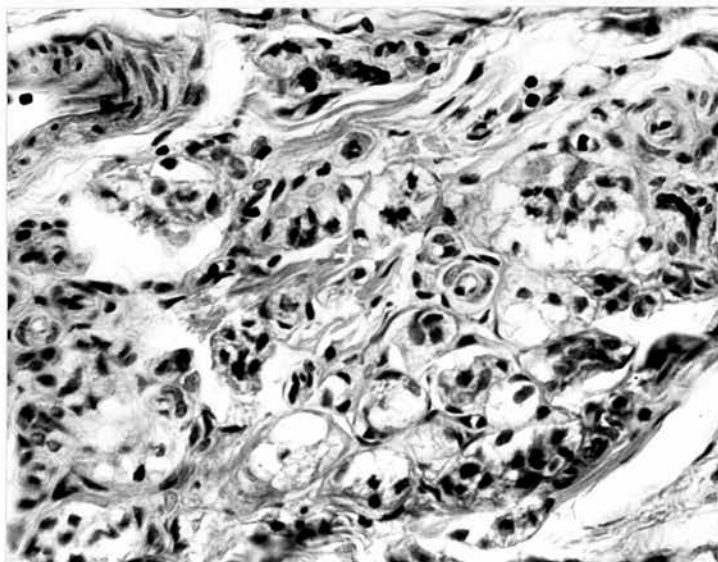


Fig. 5.

Dog 1. Sclerosed diabetic pancreas.  
Haematoxylin and Eosin. x 400.  
Atrophic acini invested by fibrous  
tissue.



EXPLANTATION OF SCLEROSSED ALLOXAN-DIABETIC PANCREAS.Experiment 1.

14.11.52: Sclerosed pancreas from an alloxan diabetic dog (Dog 1.) was removed as described in Section II of this thesis. The sclerosed portion comprised rather less than half of the animal's pancreas. A substantial part of this was immediately placed in sterile Tyrode solution in a boiling tube and taken at once to the tissue culture room. The remainder was used for histology (figs. 4 and 5).

Embryo extract had been freshly prepared beforehand by the method previously described. The pancreas was placed in a petri dish and washed with more Tyrode. It was then cut with Borrowdale needles into small pieces of the size indicated as suitable in the preliminary experiments.

24 Carrel flasks (D.3.5) were set up in the type of rack illustrated (fig. 6). This rack was used because it was found convenient to have the flasks rigidly fixed, when angling the rack to flame the flask-necks. The cotton-wool plugs were removed from all the flasks and the necks were flamed. 25 drops of the plasma-Tyrode mixture of the proportions determined in the preliminary experiments were now added to all the flasks from a fine Pasteur pipette. The plasma-Tyrode was





Fig. 6.

Copper racks for holding Carrel (D.3.5)  
flasks.

spun round inside the flask to ensure that all the inner surfaces were covered, otherwise they steam up and it is impossible to see the explant. The explants were now added using a medium sized Pasteur pipette to transfer them to the flasks. (This was used because it is more convenient than a platinum loop and does not injure the explants as a needle would). Only 3 flasks at a time have this done, as sometimes, particularly if one waits too long with fresh plasma, the explant itself will cause the plasma to clot, and it is undesirable to have this happen until the tissue is centred in the flask. This having been done, 1 drop of 1:4 embryo extract was added and gently mixed with the plasma-Tyrode. The explant is now centred and the clot allowed to form, which it will do in a few minutes. In this way the explants were set up in flasks 1-22. Flasks 23 and 24 had no explant added as they were to serve as reagent controls in the assays.

When this had been completed, the necks of the flasks were again flamed and sterile rubber stoppers were placed in them, using forceps and only tightening the fitting by hand. Each flask was numbered with glass writing ink. They were set to incubate at 37°C. in an ordinary bacteriological incubator.

The following day a number of the cultures

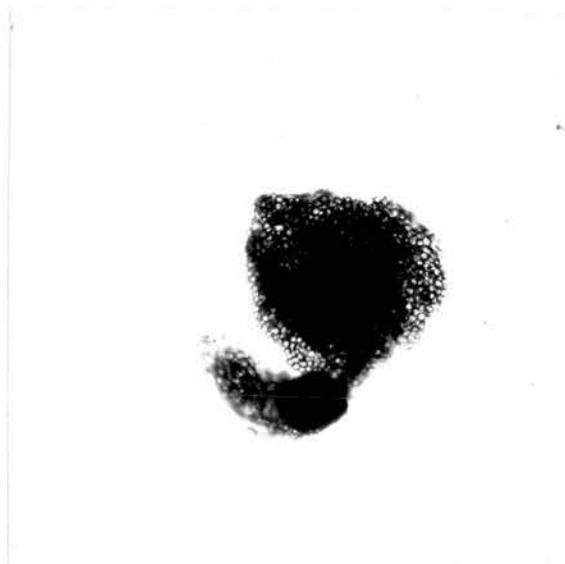


Fig. 7.

Culture 1. x 13. (in D.3.5 flask).  
24 hours after explantation. Fatty  
tissue and sclerosed diabetic pancreas.



Fig. 8.

Culture 5. x 13. (in D.3.5 flask).  
24 hours after explantation. Sclerosed  
diabetic pancreas and fat.

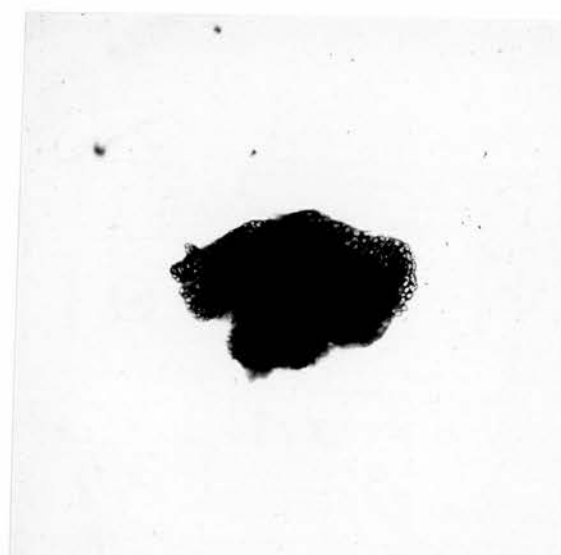


Fig. 9.

Culture 6. x 13. (in D.3.5 flask).  
24 hours after explantation. Sclerosed  
diabetic pancreas and a little fat.

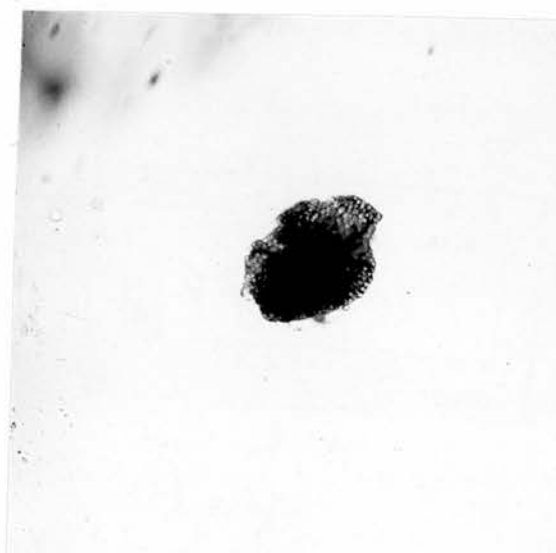


Fig. 10.

Culture 8. x 13. (in D.3.5 flask).  
24 hours after explantation. Sclerosed  
diabetic pancreas and a little fat.



Fig. 13.

Culture 17. x 13. (in D.3.5 flask).  
24 hours after explantation. Sclerosed  
diabetic pancreas and a little fat.

were photographed (figs. 7 - 13). As will be noticed, there is some opaque tissue which is probably sclerosed pancreas and should contain the surviving A-cell tissue as well as the fat which is also seen infiltrating many explants. For photographing, the flasks were inverted and held on a specially made frame which was fixed on to the stage of the microscope. The pictures were taken through the glass of the bottom of the flask.

When examined by the dissecting microscope through the glass bottom of the flask it could be seen that most of the cultures were healthy at this time. This is known by the colour and ability to transmit light. When a culture is declining, the centre darkens. This may not be appreciated on microphotographs where variations in the depth of the printing inevitably smudge such a distinction, but the grosser changes may be seen sometimes even in a photograph, by comparing for example the early and late photographs of culture 5 (figs. 8 and 15).

By the third day the clots of some cultures were beginning to liquefy so all clots were patched with cockerel plasma, Tyrode and embryo extract.

On the fourth day supernatant fluid was added to every flask.

Tyrode 0.1 c.c.  
Human serum 0.5 c.c.



Details of the appearances of the explants may be found in Appendix 1. After 18.11.52 records of only cultures 4, 5, 9, 8, 17, 19 and the reagent controls 23, 24 and later 25, 26 and 27 will be set out in Appendix 1 for reasons of brevity, as the experiment continued for a fortnight. These records are those of the explants of which the supernatant fluid was actually assayed, and those of the reagent control flasks.

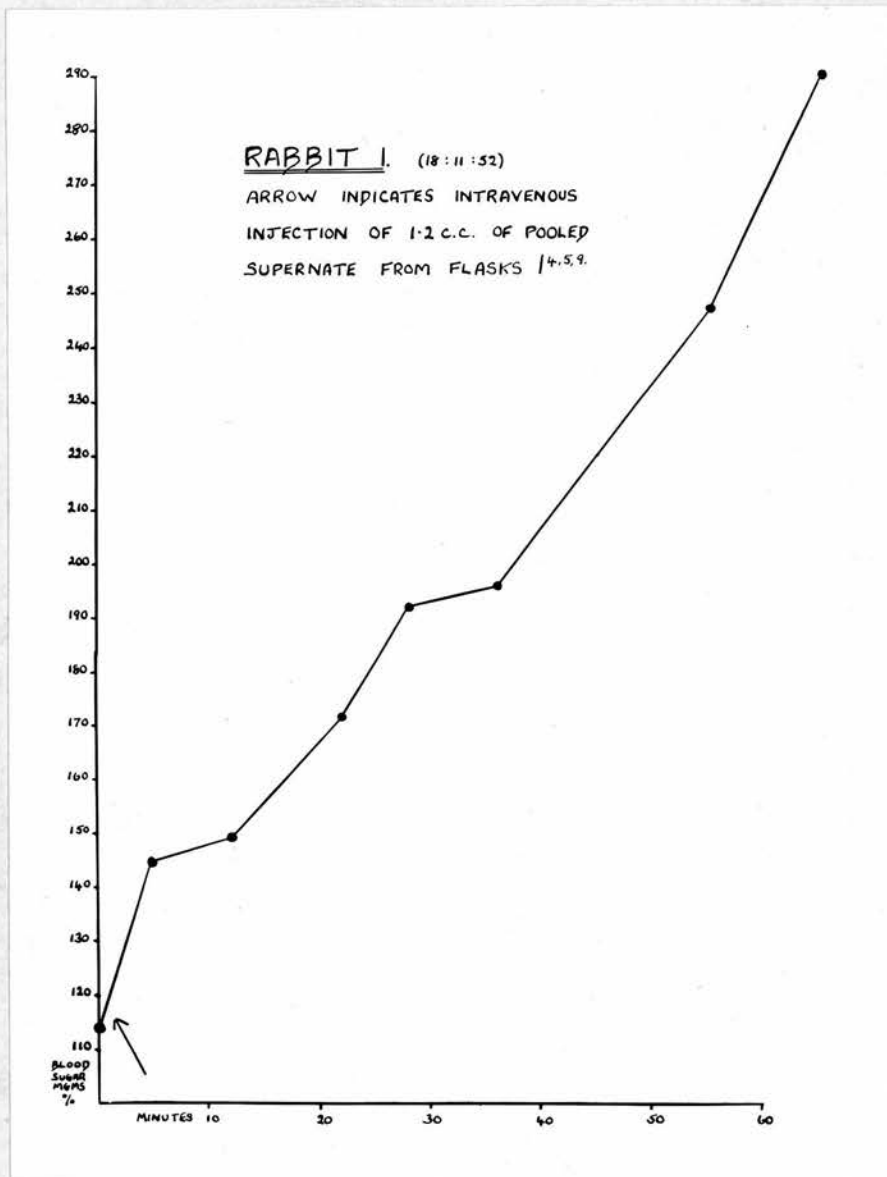
At this point it is appropriate to detail the method of assay.

Method of determining hyperglycaemic activity of supernatant fluid.

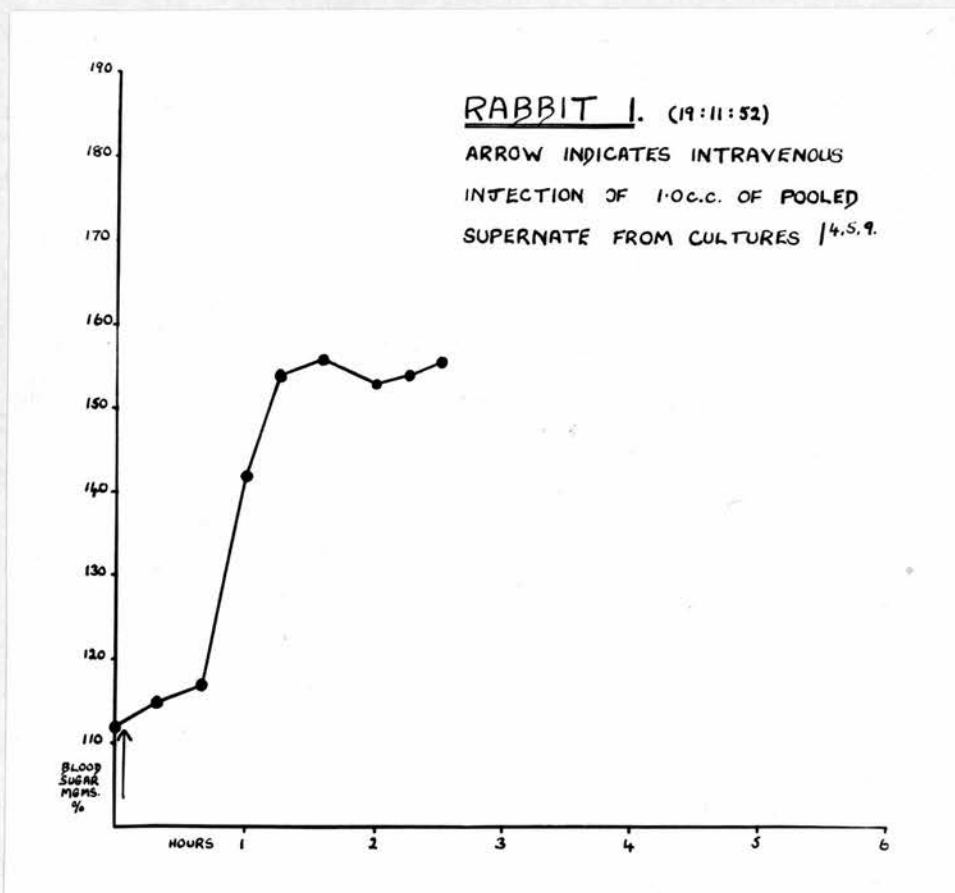
The rabbit to be used was placed in a metabolism cage the night before the assay. In the morning the urine was collected, filtered and tested for sugar by Benedict's test and for acetone by Rothera's test. The animals had access to water overnight, but not to food. Next morning the animals were placed in the rabbit box where they remained for two hours during which time a fasting blood sugar was taken, the injection given into an ear vein, and then blood for sugar estimation taken every 20 - 30 minutes. After two hours the animals were returned to the metabolism cage, where they had access to water, but not to food. Blood

for sugar was now taken hourly (approximately) up to 6 hours after injection, putting the rabbits back in the box each time. They were then returned to the metabolism cage overnight and food and water were given. The urine passed from after the first 2 hours of the test until the next morning was collected and examined for the presence of sugar and acetone. The blood sugars were estimated by the method of Hagedorn and Jensen. 0.2 m.l. of blood was taken from an ear vein into a 0.2 m.l. pipette. It was found that often considerable warmth had to be used to dilate the ear veins of the rabbit, and that this often caused the blood to clot in the pipette unless it was possible to take it very quickly. It was convenient in all the blood sugars done to draw up to the mark some Heparin (5000 units/c.c.) and then expel it again, making sure the minimum was left in the lumen of the pipette. This prevented the clotting and did not appear to have any influence on the blood sugar curves and if indeed it had any effect it would be a uniform one. The blood was put into a mixture of 2 c.c. N/10 caustic soda and 10 c.c. 0.45%  $\text{Zn} \cdot \text{SO}_4 \cdot 7\text{H}_2\text{O}$ . The specimens were taken to the Clinical Chemistry Laboratory for estimation usually within 3 hours of taking the blood.

It was generally found convenient to do 2 curves simultaneously as it avoided waiting for so



Graph 4.  
Flasks 14,5,9 signifies flasks  
4,5 and 9 of experiment 1.



Graph 5.



Fig. 14.

Culture 4. x 13. 11 days after explantation. Fat and sclerosed diabetic pancreas. Slight liquefaction of the clot is seen.

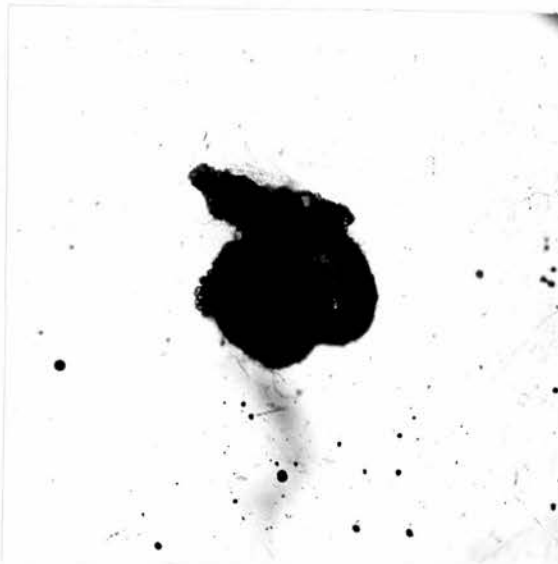


Fig. 15.

Culture 5. x 13. 11 days after explantation. This explant is considerably darker now, indicating tissue death. (c.f. fig. 8).

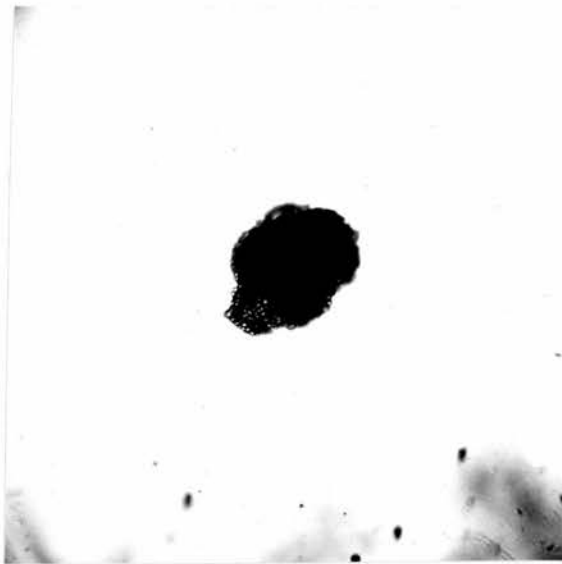


Fig. 16.

Culture 8. x 13. 11 days after explantation. Explant shows darkening and necrosis at one pole (c.f. fig.10), although some healthy tissue is still present.

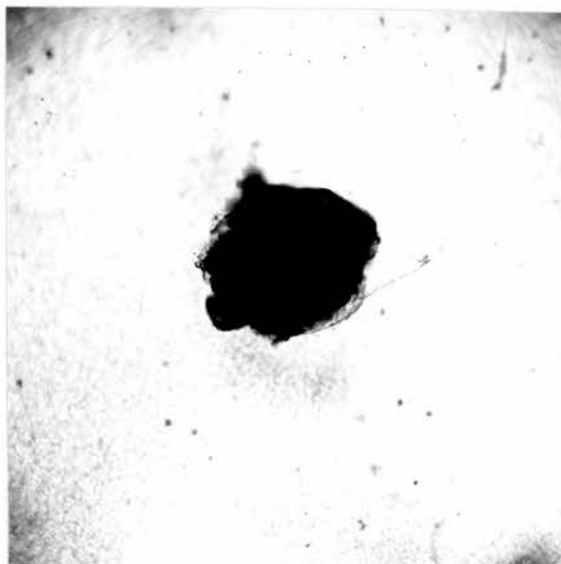


Fig. 17.

Culture 9. x 13. 11 days after explantation. Explant is darkening. Clot shows cloudy opacities and a narrow area of liquefaction near the explant.





Fig. 18.

Culture 17. x 13. 11 days after explantation. The explant is darkening.



Fig. 19.

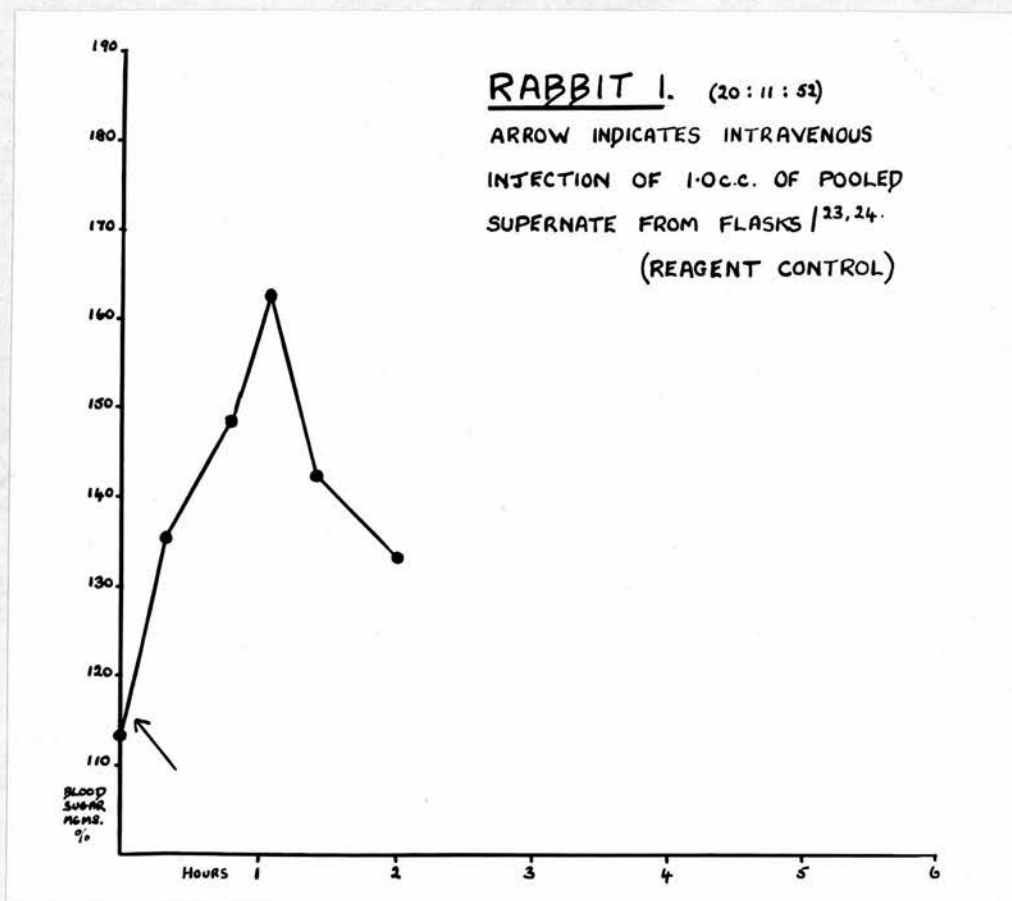
Culture 19. x 13. 11 days after explantation. Explant shows a little darkening. Some clot debris is seen and some opacity of the clot.

long between samples. In the case of the earlier curves seen on Graphs 4 - 6 it was not realised how prolonged the hyperglycaemia would be and these were taken over a shorter period. The hyperglycaemic effects of glucagon are very potent. It has been stated that a significant effect on the blood sugar of a rabbit can be obtained by injecting as little as  $10\mu$  gm. (de Duve, 1953).

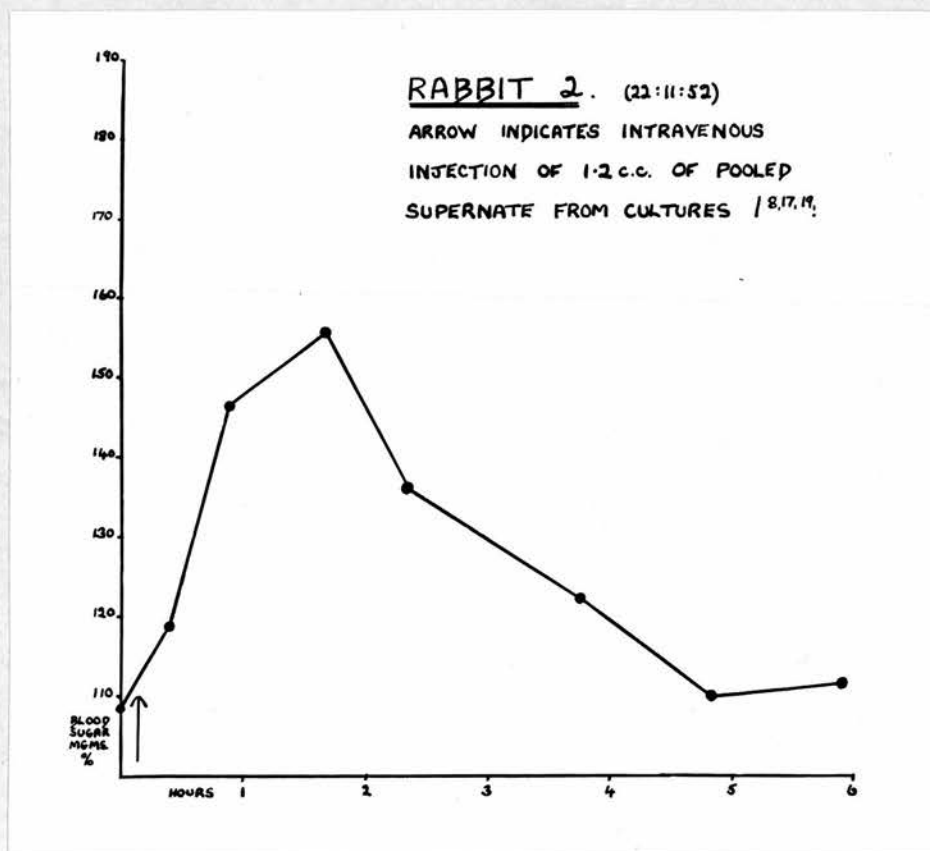
18.11.52: The effects of intravenous injection of rabbits with 1.2 c.c. of the pooled supernatant fluids from cultures 4, 5 and 9 can be seen on Graph 4. As it was not known at that time what effect to expect, the readings were taken every 5 minutes for 1 hour. Quite a remarkable rise occurred from a fasting level of 114 mgm.% to greater than 290 mgm.%.

The supernatant fluid in the flasks was replaced at once by a similar quantity of human serum and Tyrode and the flasks put again to incubate. This was done throughout, whenever a supernate was removed for injection.

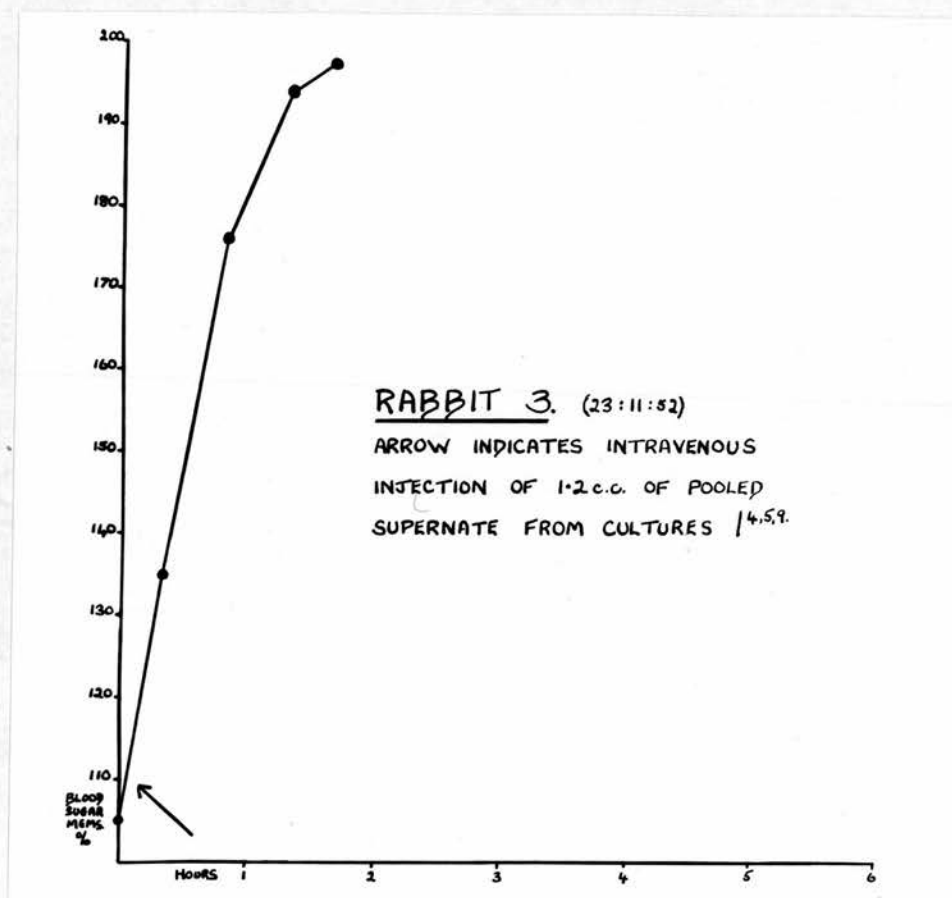
On the following day, after a night's incubation, 1 c.c. of pooled supernate was again injected into rabbit 1. A significant effect was produced, but much less marked than the first one. It lasted for over  $2\frac{1}{2}$  hours. (c.f. Graph 5).



Graph 6.



Graph 7.



Graph 8.

The next day (20.11.52) the effect of injecting the supernate from the reagent control flasks was tried. This produced an effect similar to that on Graph 5, but of shorter duration. (c.f. Graph 6).

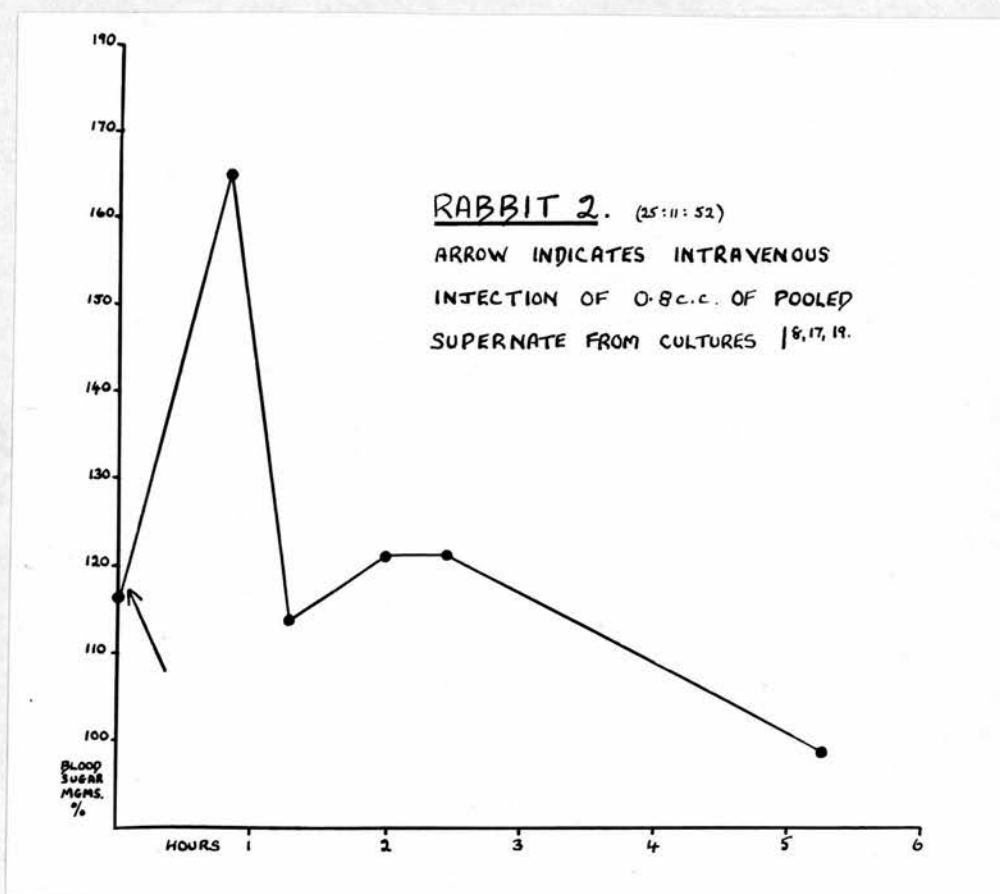
It was now decided to extend the readings over 6 hours.

On 22.11.52, 1.2 c.c. of the pooled supernatant serum from cultures 8, 17 and 19 was injected into rabbit 2 and quite a marked response resulted (c.f. Graph 7), but not so marked as after the injection of the first supernate from cultures 4, 5 and 9 (c.f. Graph 4).

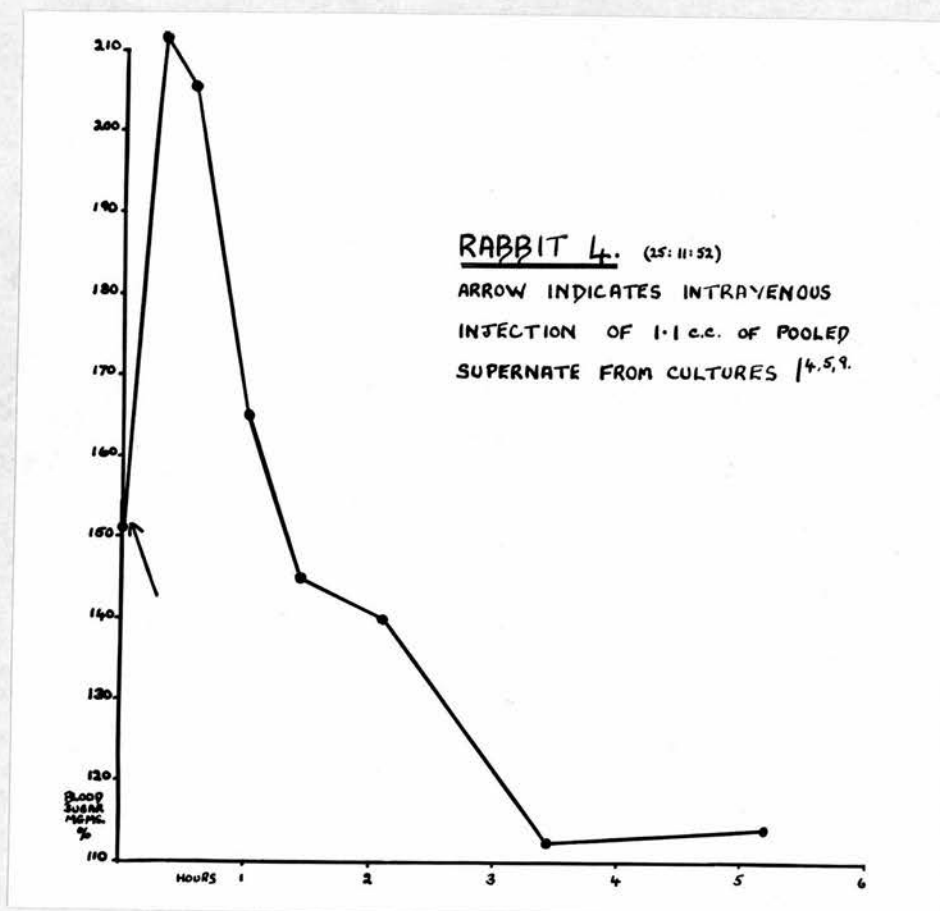
On 23.11.52, 1.2 c.c. of the third supernate of the cultures 4, 5 and 9 was given to Rabbit 3 after having been incubated with the explant for 4 days. The result is another very striking rise in blood sugar. Unfortunately, this curve could not be continued beyond 100 minutes because the rabbit's veins were difficult and the animal objected vigorously. However, even in this time the blood sugar rose from 105 mgm.% to 198 mgm.%. (c.f. Graph 8). In none of these instances was sugar obtained in the urine, and it was not obtained in any such assays recorded in this thesis.

By 20.11.52 a few of the explants were dead and a few showed necrotic areas, but most were





Graph 9.



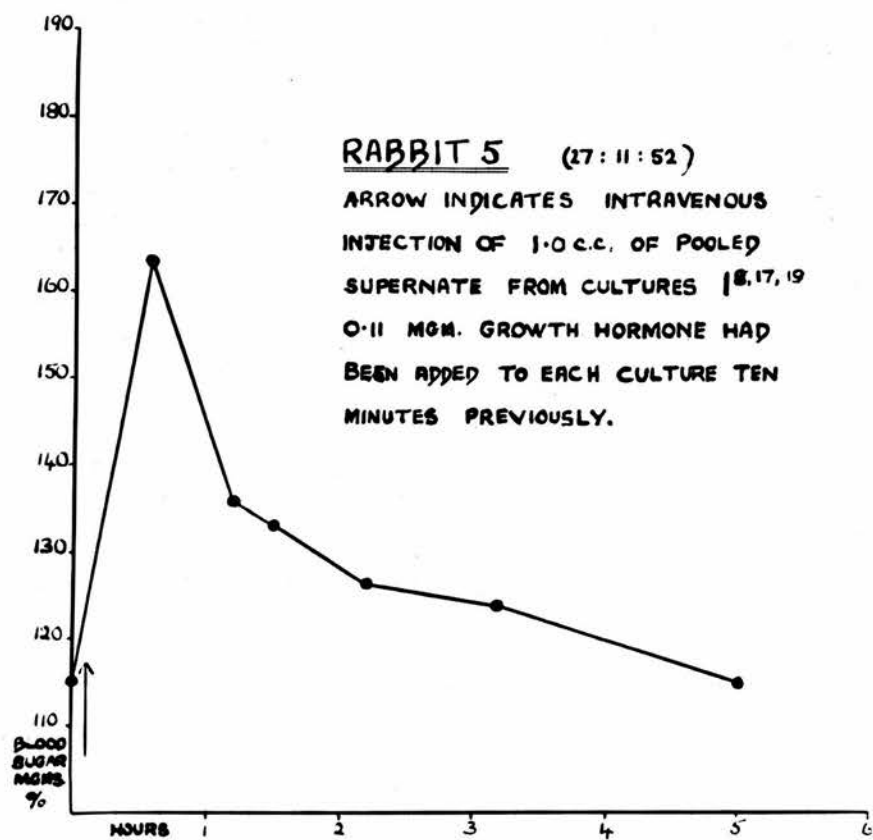
Graph 10.

reasonably healthy. Growth was either not observed or was very slight as had been intended. Cultures 4, 5 and 9, 8, 17 and 19 were the most healthy and for that reason had been selected for assaying. Supernatant fluids were changed as indicated in Appendix 1.

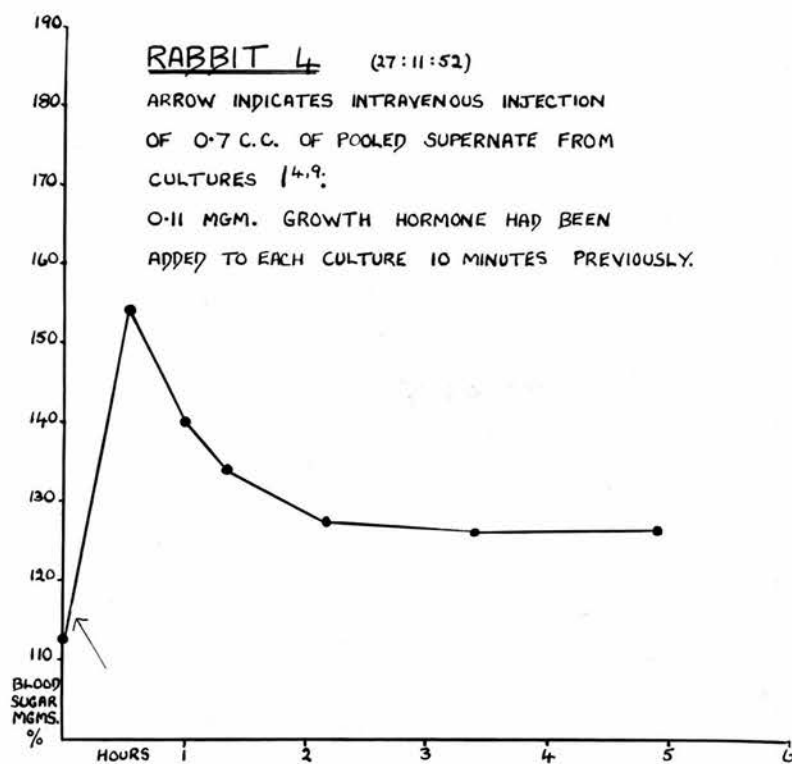
By 23.11.52 some of the explants were showing central necrosis, but were alive at the edges. The cultures being assayed continued to thrive.

On 25.11.52, 0.8 c.c. of pooled supernate from cultures 8, 17 and 19 was injected into Rabbit 2 and a marked, but short-lived effect was produced. (c.f. Graph 9). At the same time Rabbit 4 had an injection of 1.1 c.c. of pooled supernate from cultures 4, 5 and 9. This was the fourth supernate these cultures had had, and had been incubated with them for 2 days. The effect was marked, the blood sugar rising from 151 mgm.% to 212 mgm.% in 20 minutes and then falling sharply. (c.f. Graph 10).

At this time cultures in use were photographed (4, 5, 9, 8, 17 and 19) and it was found the clot had broken loose from 5 and 19. These explants were removed from their flasks and washed in Tyrode solution. They had the old clot trimmed off and were put up in a fresh clot. (figs. 14 - 19).



Graph 11.



Graph 12.



GROWTH HORMONE.

In view of the findings of Bornstein, Reid and Young (1951) that growth hormone provoked an immediate secretion of a hyperglycaemic substance into the portal blood of an A.D.H.A. rat, it was thought it would be of some interest to add a quantity of growth hormone to the cultures and see if a more marked hyperglycaemic effect than already seen could be elicited by such a possible excitation of A-cells.

It was difficult to know a suitable starting dose of growth hormone to add to each flask. Initially the dose was arbitrarily fixed at 0.11 mgm. crystalline growth hormone (Wilhelmi).

(27.11.52) This was given in 0.3 c.c. of Tyrode solution, the growth hormone was incubated with the culture for 10 minutes, then the supernate was withdrawn from the flasks and injected. (Bornstein, Reid and Young (1951) found the maximum effect of the growth hormone in the A.D.H.A. rat was observed 10 minutes after injecting it).

As can be seen from Graph 11 and Graph 12, a hyperglycaemic effect is produced no different from many of the other curves.

About this time (27.11.52) it was found that fungus infection had got into some of the flasks. This was due probably to the repeated withdrawals

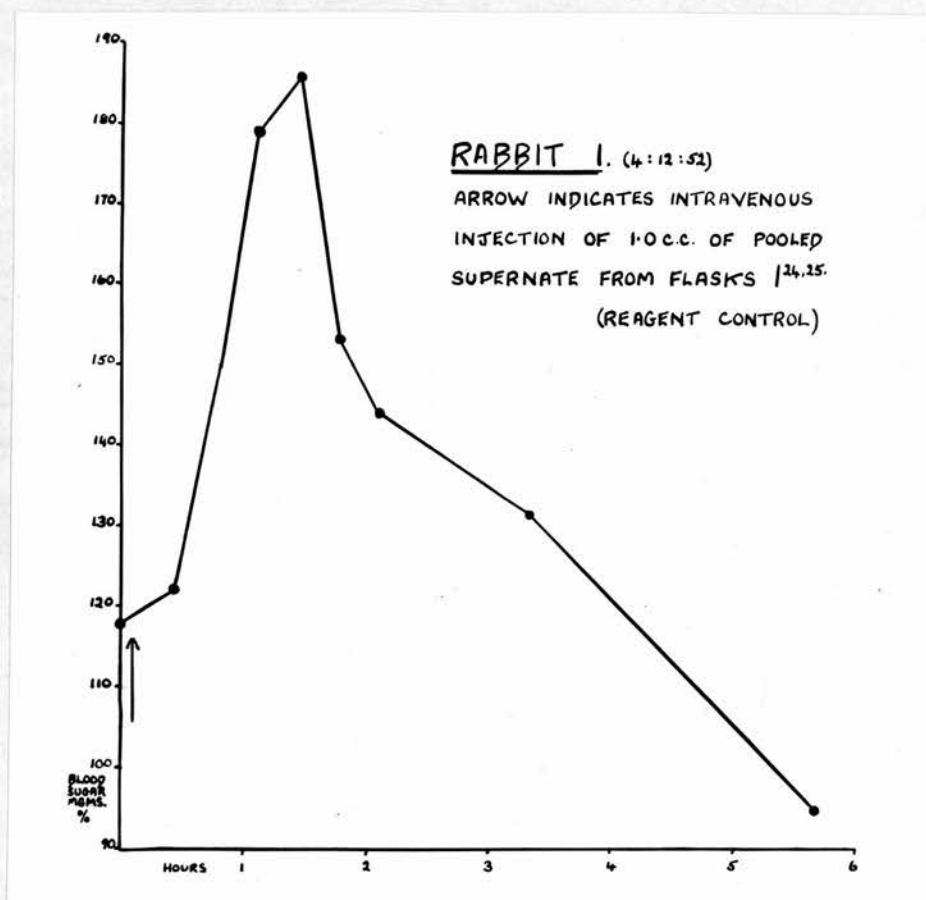


of supernate from them. This spread to other flasks and as many of the explants now looked rather necrotic the culture part of the experiment was ended, and on 30.11.52 the explants were sent for section. It was not, therefore, possible to go on to vary the doses of growth hormone. The reagent control flasks were retained in the incubator, and later the clots were replaced in these. The sections of these explants showed they consisted of connective tissue and fat which was largely necrotic. Preservation was not good enough to identify pancreatic tissue, although the sections seen on figs. 4 and 5 indicate that sclerosed pancreas was present in the tissue from which the explants were derived.

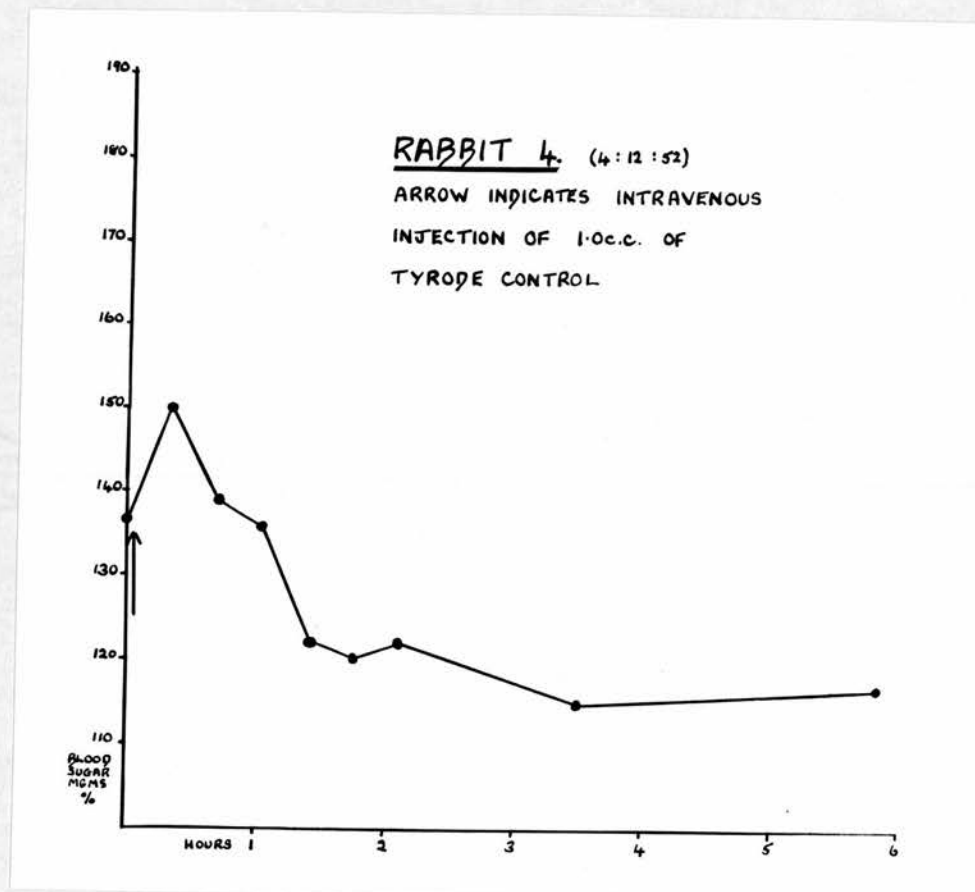
#### CONTROLS.

Apart from one injection of reagent control supernate (Graph 6) no controls were done during the explantation experiment, because it was desired to do as many blood glucose curves after injection of culture supernate as possible, while the explants were still alive.

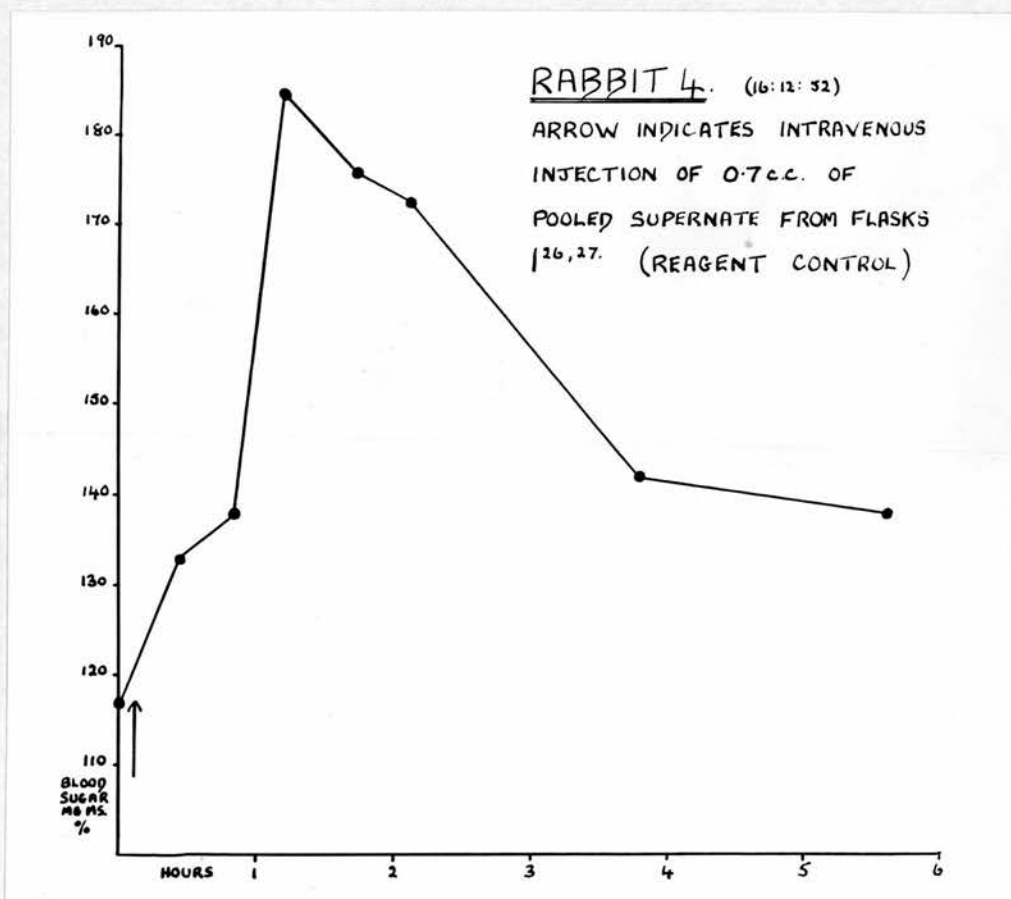
The controls done now were Reagent controls, i.e. flasks containing the same media as the pancreatic explants and incubated



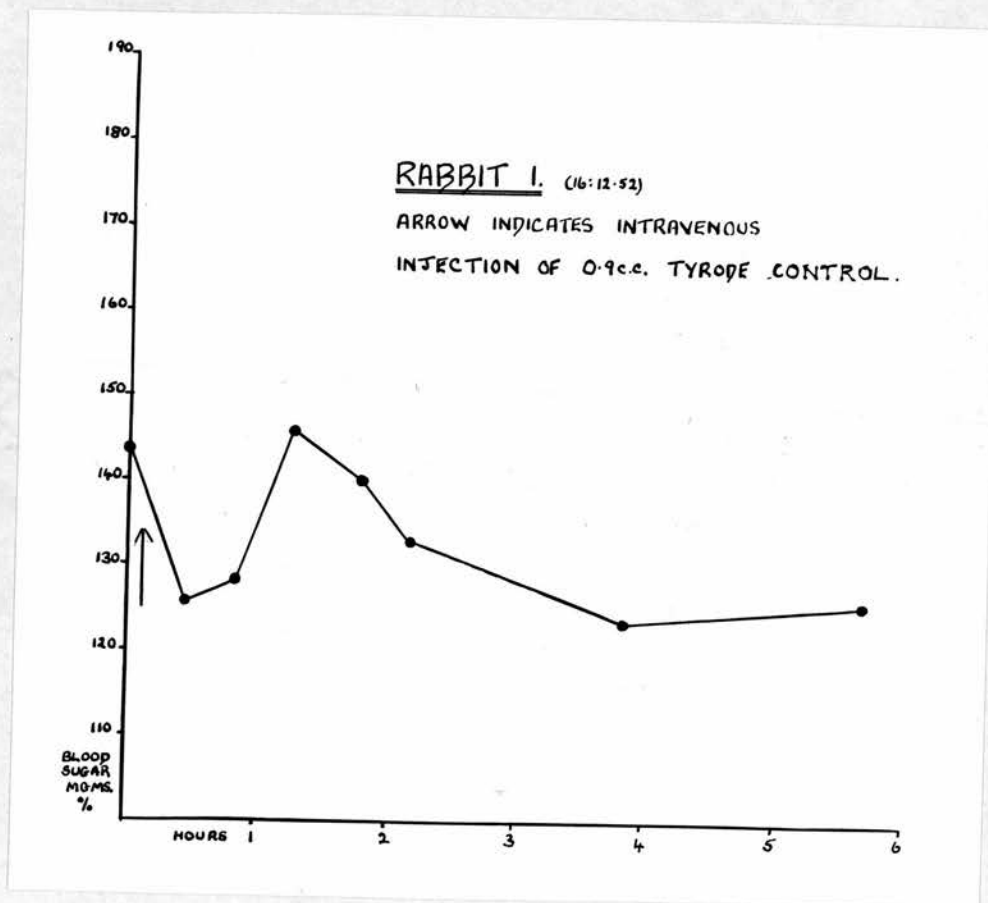
Graph 13.



Graph 14.



Graph 15.



Graph 16.

for at least the same length of time, but containing no explant. The results of injection of such supernates are seen on Graphs 6, 13 and 15.

#### Tyrode Controls.

Tyrode solution is a physiological saline solution, non-proteinous in nature. The results of injecting it are seen in Graphs 14 and 16.

It is evident that the serum supernates constituting the reagent controls produce a type of blood sugar curve in the rabbit indistinguishable from that produced by the same fluid when a culture was present, but with certain exceptions.

A curve of the height seen in Graph 4 was never produced without a pancreatic explant. A type of curve similar to this, but unfortunately incomplete, was produced by supernate from the same cultures (4,5 and 9), on a different rabbit. (c.f. Graph 8). The same cultures (4,5 and 9) produced another very high curve (c.f. Graph 10). on yet another rabbit. Here, however, it is not so easy to know how much is due to serum and how much to a possible hyperglycaemic factor. These 3 curves were performed respectively 4, 9 and 11 days after explantation.



Graph 5 is not the high type of curve sometimes seen with cultures 4, 5 and 9. It is thought this may be due to the fact that the supernate had been changed only the day before and that would have removed any possible hyperglycaemic factor for the time being.

Cultures 8, 17, 19 did not show such high curves. It is possible that they contained less A-cell tissue than cultures 4, 5 and 9, and that any hyperglycaemic effect produced by an A-cell substance was entirely obscured by the serum effect.

The Tyrode injections showed no appreciable effect on the blood sugar at all.

It seems there are probably 2 types of curve. One tending to occur earlier in the culturing, possibly due to the secretion of a hyperglycaemic substance and disappearing later, and one which is apparently due to the presence of serum, for it is produced by the supernatant fluid placed in the cultures. As this fluid consists chiefly of human serum with a little Tyrode, and the Tyrode, a non-proteinous solution, produces no such effect, then it is reasonable to suppose that the effect is due to the serum. Although this curious serum effect was not strictly relevant to the main thesis, the effects of various proteins on the blood sugar

curves of rabbits were investigated to some extent, and the results are recorded in Appendix iii.

The 2 types of curve mentioned will inevitably be superimposed on each other in this type of experiment, and it is difficult to know how much is due to the possible secretion of glucagon, by the residual A-cells, and how much to the serum, though it can be said that these early curves (c.f. Graphs 4, 8 and possibly 10) may indicate something additional to the serum effect.

PEPTONE CONTROL.

It was suggested that the hyperglycaemic effect ascribed to the serum might be due to the formation of peptones on incubating the protein of the reagents and that these might cause hyperglycaemia. Accordingly it was resolved to compare the effects of dialysed unincubated serum showing no trace of peptone, with an injection of the same quantity of the same serum which had been incubated for varying lengths of time.

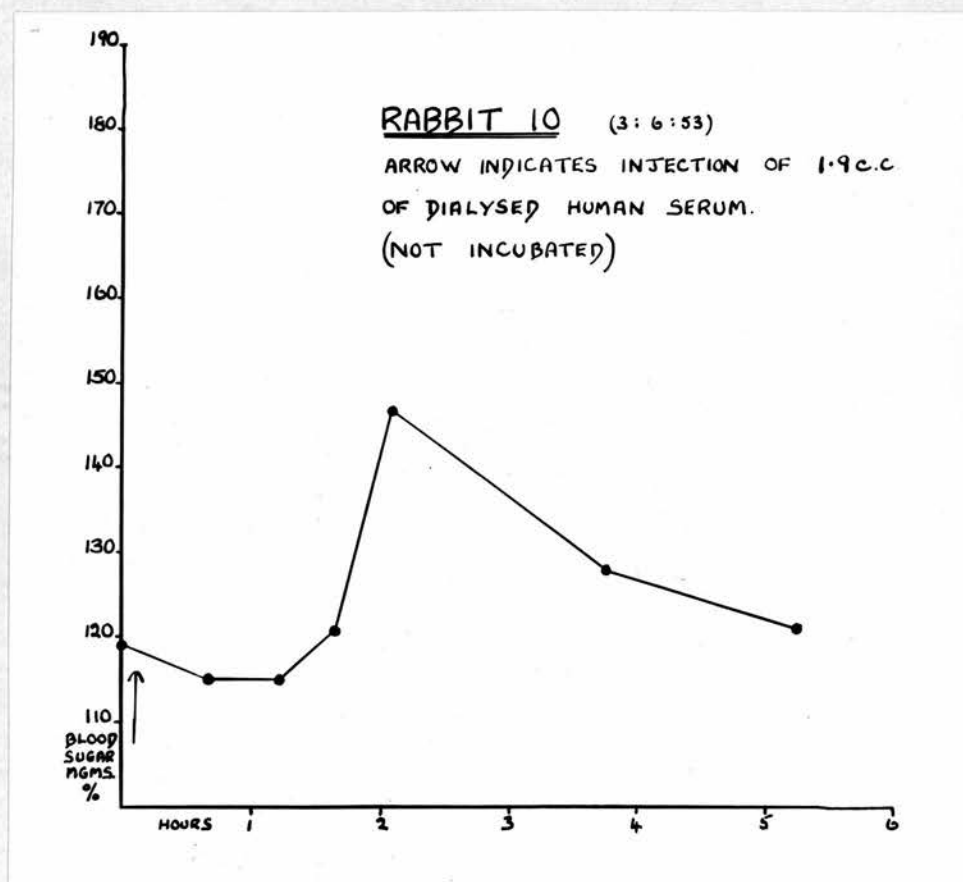
20 c.c. of human serum were put in a cellophane 'sausage skin' and left to dialyse in running water for 72 hours. The serum protein and non-protein nitrogen (N.P.N.) were then estimated and it was seen the protein concentration had fallen.

Albumen	2.37 G. per 100 c.c.
Globulin	1.6 G. per 100 c.c.
N.P.N.	18 mgm. per 100 c.c.

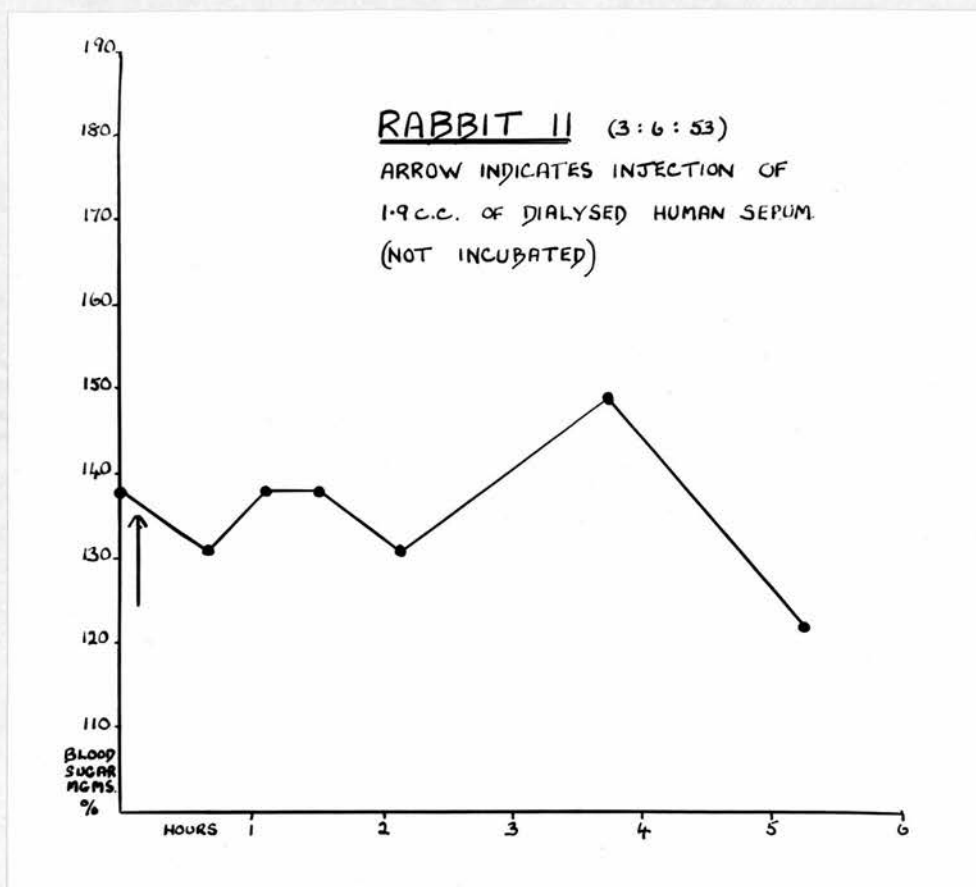
These results were compared with ones obtained from a specimen of the same serum which had not been dialysed.

Albumen	4.21 G. per 100 c.c.
Globulin	2.52 G. per 100 c.c.
N.P.N.	24 mgm. per 100 c.c.

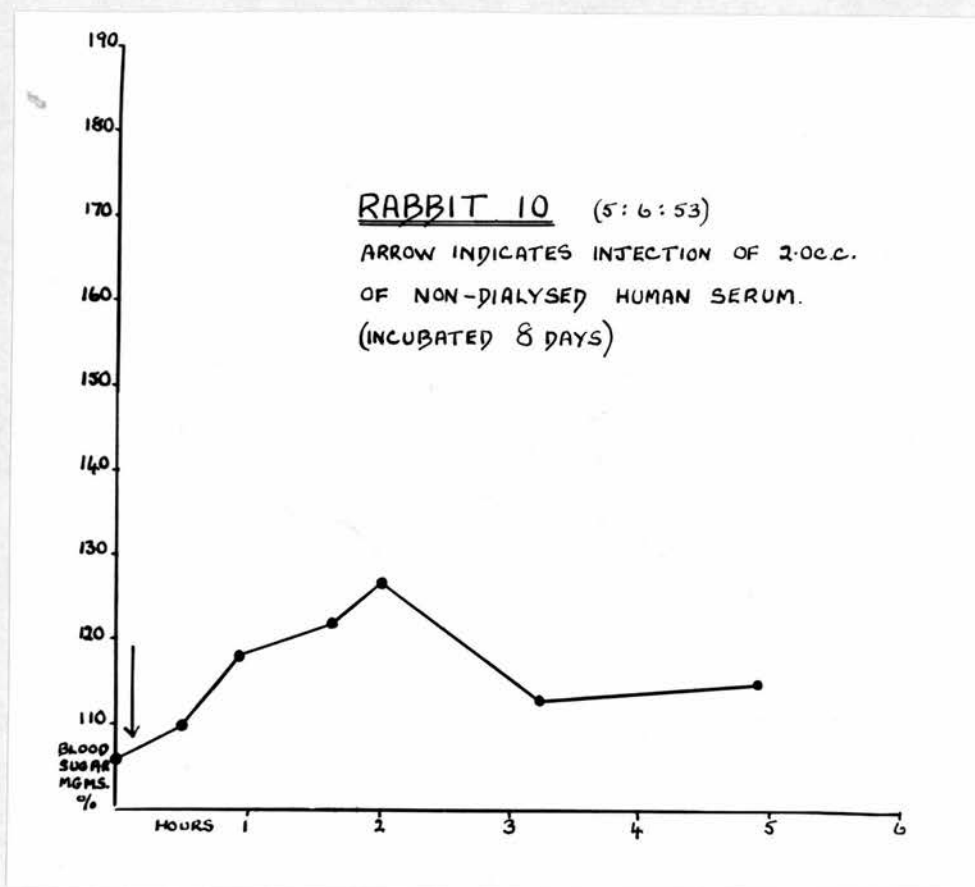
To ensure the quantity of protein injected in each case was the same, the undialysed serum



Graph 17.

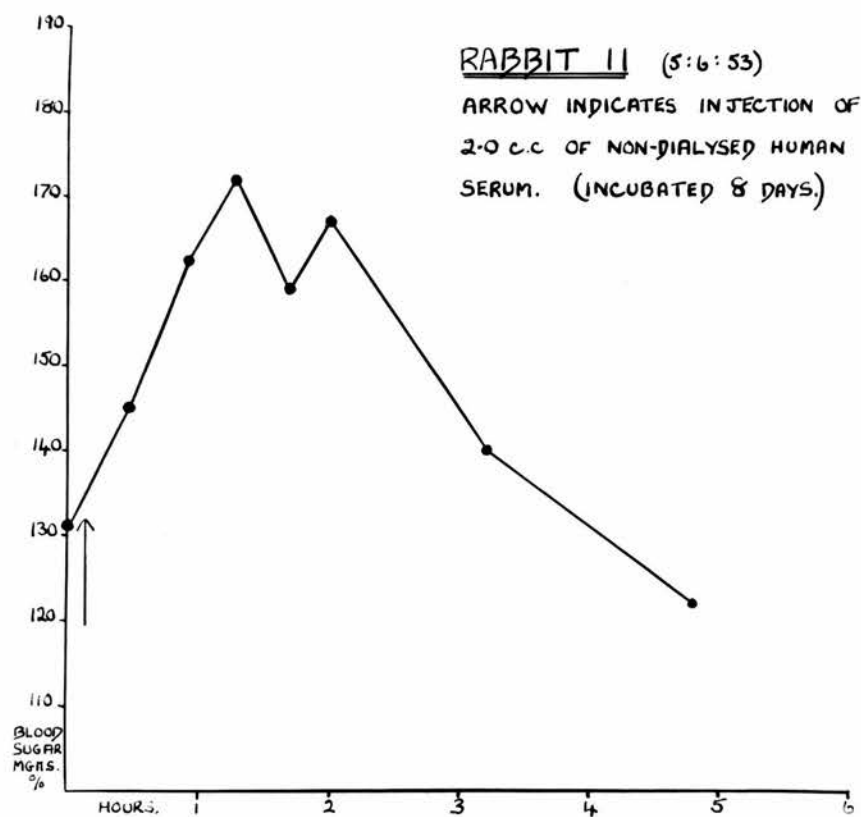


Graph 18.

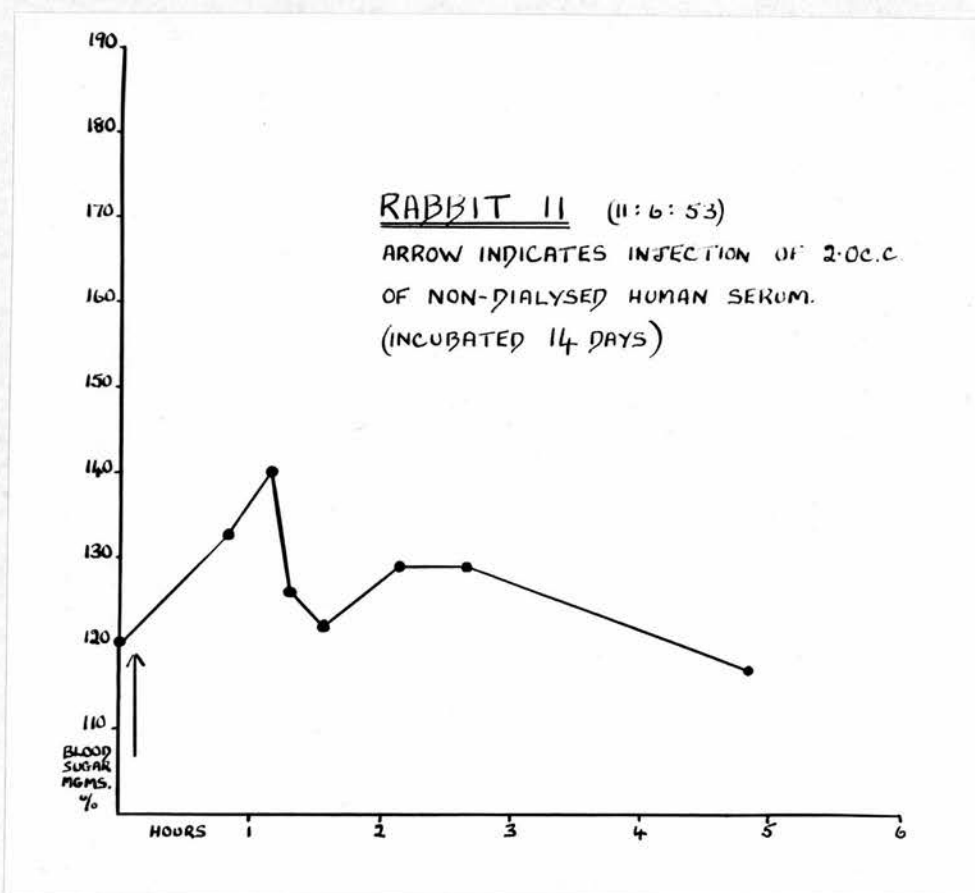


Graph 19.





Graph 20.



Graph 21.

was diluted until the total protein was equal to that of the dialysed serum.

A Biuret qualitative test for peptone (Cole, 1941) in the dialysed serum showed NO trace of peptone. The dialysed serum was stored at  $-25^{\circ}\text{C}$ . until required for injection. The diluted non-dialysed serum was incubated at  $37^{\circ}\text{C}$ .

1.9 c.c. of dialysed, non-incubated serum was given intravenously to each of 2 rabbits, and the blood sugars estimated over 5 - 6 hours (c.f. Graphs 17 and 18).

The same 2 rabbits each had 2 c.c. of 8 day incubated, non-dialysed serum intravenously and the blood glucose curves were estimated (c.f. Graphs 19 and 20). After 14 days incubation, again the same 2 rabbits (Rabbits 10 and 11) had 2 c.c. of the non-dialysed serum. Unfortunately Rabbit 10 broke its neck struggling in the box. The other rabbit yielded a satisfactory blood glucose curve. (c.f. Graph 21).

Comparison of the effects of the dialysed serum, and the effects of the non-dialysed serum incubated for different periods, shows no significant difference. A more marked hyperglycaemic effect is certainly noted in Rabbit 11 with 8 day incubated non-dialysed serum than with the dialysed serum (c.f. Graphs 18 and 20). The curve, however,

is not sustained, and when the same rabbit had an injection of the 14 day incubated serum the effect was smaller, when if peptone had been the causative factor it should have been greater (c.f. Graph 21).

It is therefore concluded that peptone was not the cause of the hyperglycaemia noted on injecting the supernates of the tissue cultures intravenously into rabbits.

#### Summary

(Experiment 1).

- (1) Hyperglycaemic effects were produced in rabbits by the injection of the supernatant fluids from biphasic Carrel flask cultures of sclerosed alloxan diabetic pancreas.
- (2) These effects in the early part of the experiment were rather more pronounced than were obtained by injecting serum-Tyrode supernate controls. This suggests the possibility that secreting A-cell tissue was present in the explants in the first few days and that the A-cells then died, leaving only the serum effect.
- (3) Unfortunately, due to the fact that the experiment lasted over 2 weeks and the explants became necrotic, histological evidence of the presence of A-cell tissue was not obtained and islets were not identified in the sclerosed pancreas.

(4) The serum effect was not due to peptone production in the serum.

(5) This experiment is no more than suggestive that A-cells were present in the explants at first and that they secreted into the medium. It is thought, however, another experiment with improvements suggested by the experience of this first attempt may clarify the position.

EXPLANTATION OF SCLEROSSED ALLOXAN DIABETIC PANCREAS.Experiment 2.Introduction.

The results of experiment 1, while suggestive of secretion of a hyperglycaemic substance by the sclerosed diabetic pancreas were not conclusive.

It was considered in view of the findings in experiment 1 that (1) the A-cells probably would not survive long in cultures which were static and not multiplying, and that the experiment should last only a few days.

(2) To diminish the possibility of failing to include cultures which contained A-cells, that the supernatant fluids of all the cultures should be pooled and the injections prepared from this.

(3) That to eliminate the hyperglycaemic activity of the serum proteins that the possible glucagon in the pooled culture supernates should be extracted leaving the serum proteins behind. The method of extraction was an acid-alcohol one used by Sutherland and de Duve (1948), and Odée (1950), but modified to extract serum instead of solid pancreas. This method would specifically extract glucagon and any insulin remaining, but the serum proteins would be separated off (vide infra.).



(4) That there should be more adequate histological studies made to demonstrate the presence of A-cell islets in the sclerosed diabetic pancreas and in the explants. In experiment 1 the cultures were maintained too long and they became necrotic.

(5) That the pancreatic explantation should not be done so long after ligation as was done in experiment 1, as it was thought this might cause a decrease in the islet tissue by vascular atrophy and fatty replacement. In experiment 1 the period was 5 months. It was now proposed that it should be only 6 weeks.

(6) That as controls, extracts should also be made of a) the supernates from flasks where no explant had been added, and b) supernates from cultures of chick heart. This latter to exclude the possibility that the hyperglycaemic factor was a tissue metabolite.

As detailed in Section II great difficulty was experienced in preparing another alloxan diabetic dog with a sclerosed pancreas, but one was finally obtained (Dog. 16).

20.10.53: The sclerosed pancreas from the alloxan diabetic dog was removed aseptically and transferred to a boiling tube charged with Tyrode solution. The cultures were set up in D.3.5 Carrel flasks in exactly the way detailed in experiment 1, except 2 new 'Eveready' razor blades were used for cutting the explants instead of the Borrowdale needles. Pancreas was placed in 24 flasks, chick heart in 12 flasks, and a further 12 flasks contained all the reagents, but no explant.

On the following day the cultures of pancreas were healthy, but had liquefied the plasma clots. It was considered that the cockerel serum thus produced, would contain a good deal of glucagon, if any had been secreted. Therefore, it was removed from the flasks, pooled and stored at  $-25^{\circ}\text{C}$ . until it could be extracted. 6 explants were sacrificed for histology. The clots were now patched, putting 25 drops of 2:1 cockerel plasma-Tyrode mixture into each of the remaining flasks and then adding 1 drop of 1:4 embryo extract and Tyrode solution to cause clotting. The explants all looked healthy (figs. 20 - 22).

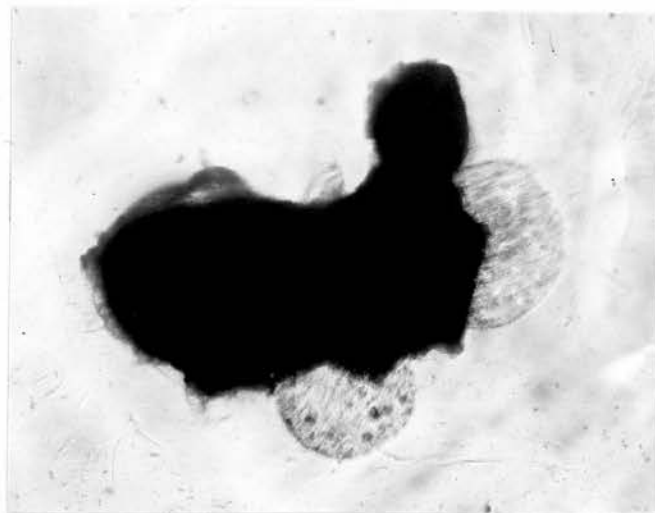


Fig. 20.

Culture P.22. x 15. 24 hours after explantation. Healthy, sclerosed diabetic pancreas. Some liquefaction of the clot is seen.

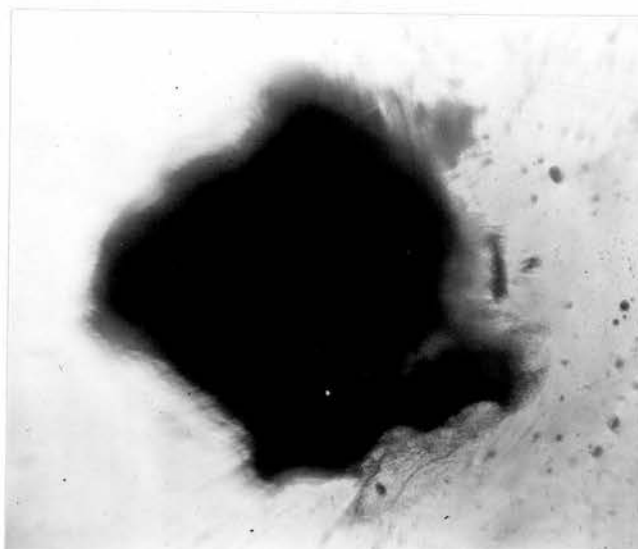


Fig. 21.

Culture P.23. x 15. 24 hours after explantation. Healthy, sclerosed diabetic pancreas.

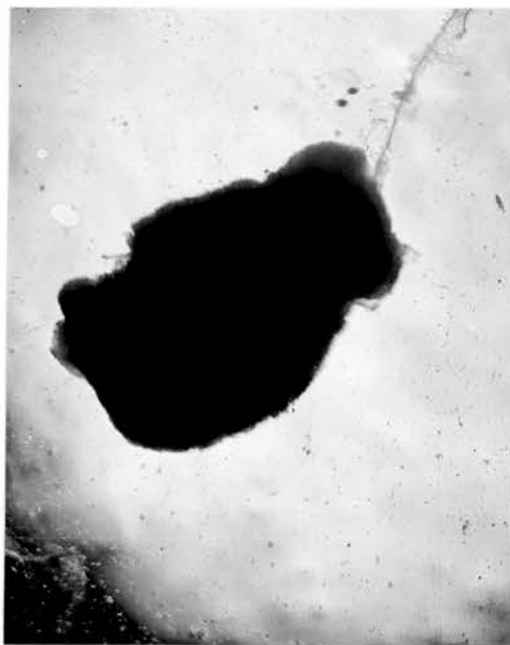


Fig. 22.

Culture P.24. x 15. 24 hours after explantation. Healthy, sclerosed diabetic pancreas.

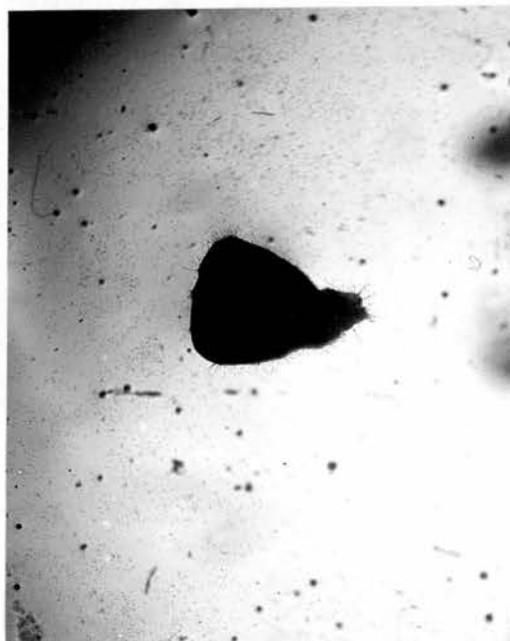


Fig. 23.

Culture H.2. x 15. 24 hours after explantation. Chick heart explant. Early fibroblastic proliferation.

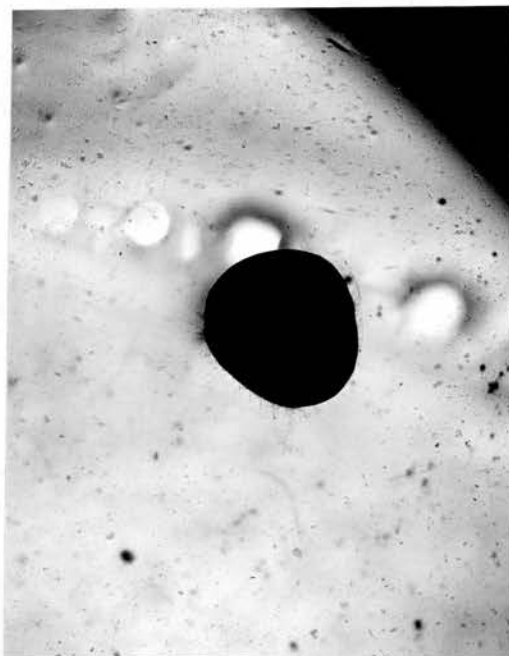


Fig. 24.

Culture H.3. x 15. 24 hours after  
explantation. Chick heart explant.  
Early fibroblastic proliferation.

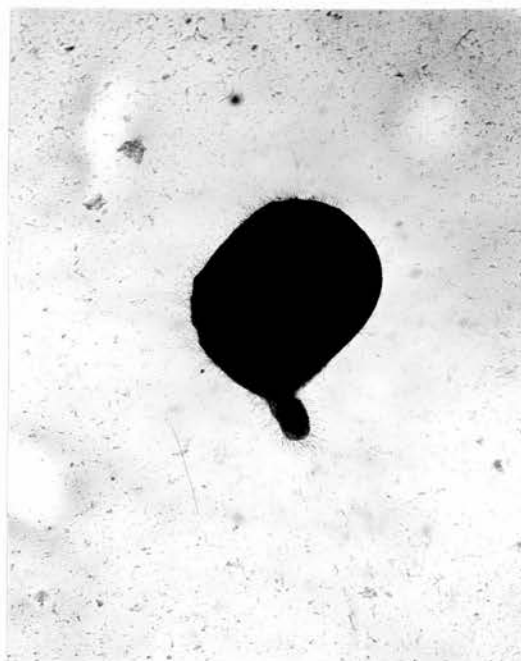


Fig. 25.

Culture H.6. x 15. 24 hours after  
explantation. Chick heart explant.  
Early fibroblastic proliferation.

The heart cultures were all healthy and the heart muscle was beating. A little fibroblastic outgrowth is seen. (figs. 23 - 25).

The explants still looked well on 22.10.53; the supernatant fluid was added to all the cultures (15 drops of 1:4 Tyrode and human serum), including pancreatic and chick heart explants and reagent control flasks. 6 more pancreatic explants were sacrificed for histological study leaving 12.

The explants were incubated with the supernatant fluid for 2 days, and the fluid from the pancreas cultures was then withdrawn and pooled. The fluid was likewise withdrawn and pooled from the reagent control flasks and the heart culture flasks.

The tubes of pooled supernates were stored at  $-25^{\circ}\text{C}$ . until they were sent to be extracted. (More details of this experiment can be seen in Appendix 1).

All the explants were fixed in 10% formol saline for histological section. (Reports on these sections will be found in Appendix 1).



GLUCAGON EXTRACTIONS OF SUPERNATANT FLUID.

The glucagon extractions were kindly performed by Dr. Gilchrist of the Department of Clinical Chemistry, Royal Infirmary, Edinburgh.

24.10.53: The 4 tubes of pooled supernatant fluids were sent to Dr. Gilchrist. They were

- (1) Pooled supernate from the pancreatic cultures taken on the second day of incubation;
- (2) Pooled supernate from the pancreatic cultures taken on the fifth day of the experiment (i.e. 2 days after incubating with the supernatant fluid);
- (3) Pooled supernatant fluid from the chick heart cultures taken on the fifth day of the experiment (i.e. 2 days after incubating with the supernatant fluid);
- (4) Pooled supernatant fluid from the reagent control flasks taken on the fifth day of the experiment (i.e. 2 days after incubating with the supernatant fluid).

The extractions were performed as detailed below:

Method of Extracting Glucagon.

This method is modified from Odée(1950) which is essentially the method of Sutherland and de Duve (1948).

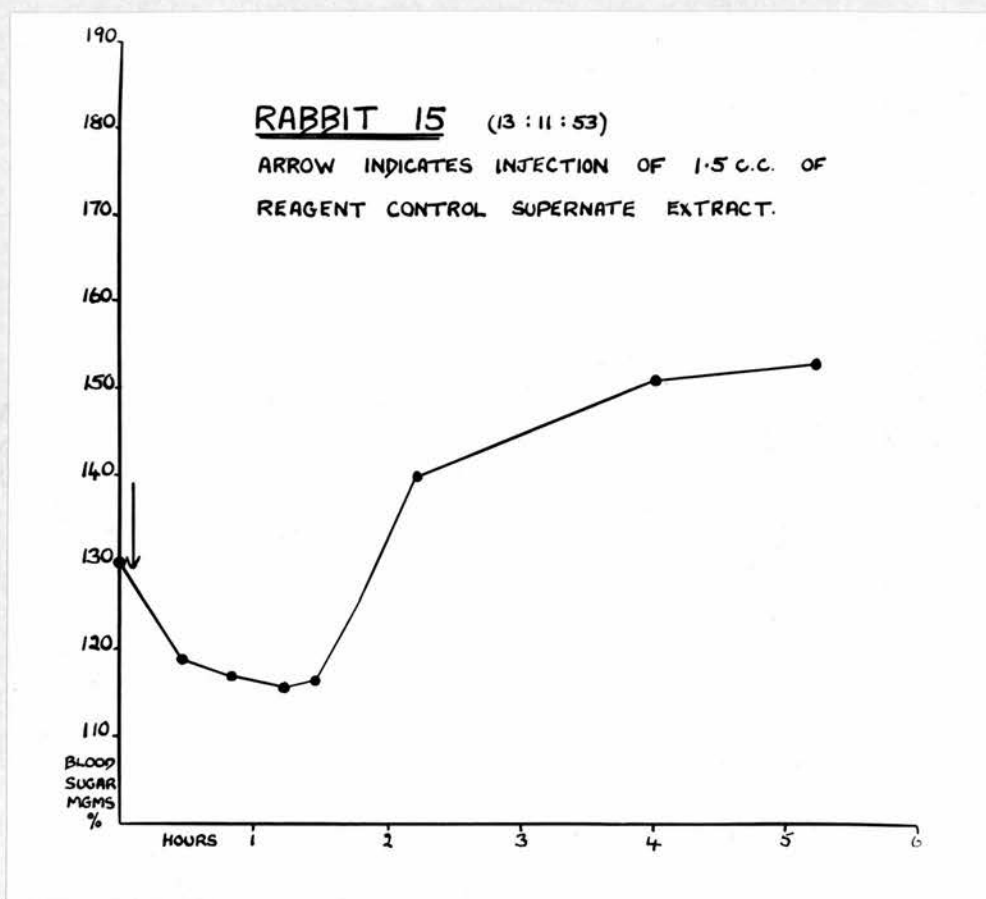
Measure an exact volume of pooled supernatant

fluid into a tube or bottle with a ground glass stopper. Add 3 volumes of 95% ethyl alcohol, acidified with HCl approximately 0.18N.\* Stopper the tube, fix it on a shaker and extract overnight at 2°C. Centrifuge and pour off the supernatant liquid. Discard the precipitate. Adjust the pH of the supernatant fluid to pH 7.4 with dilute  $\text{NH}_4\text{OH}$ . Centrifuge and discard the precipitate.

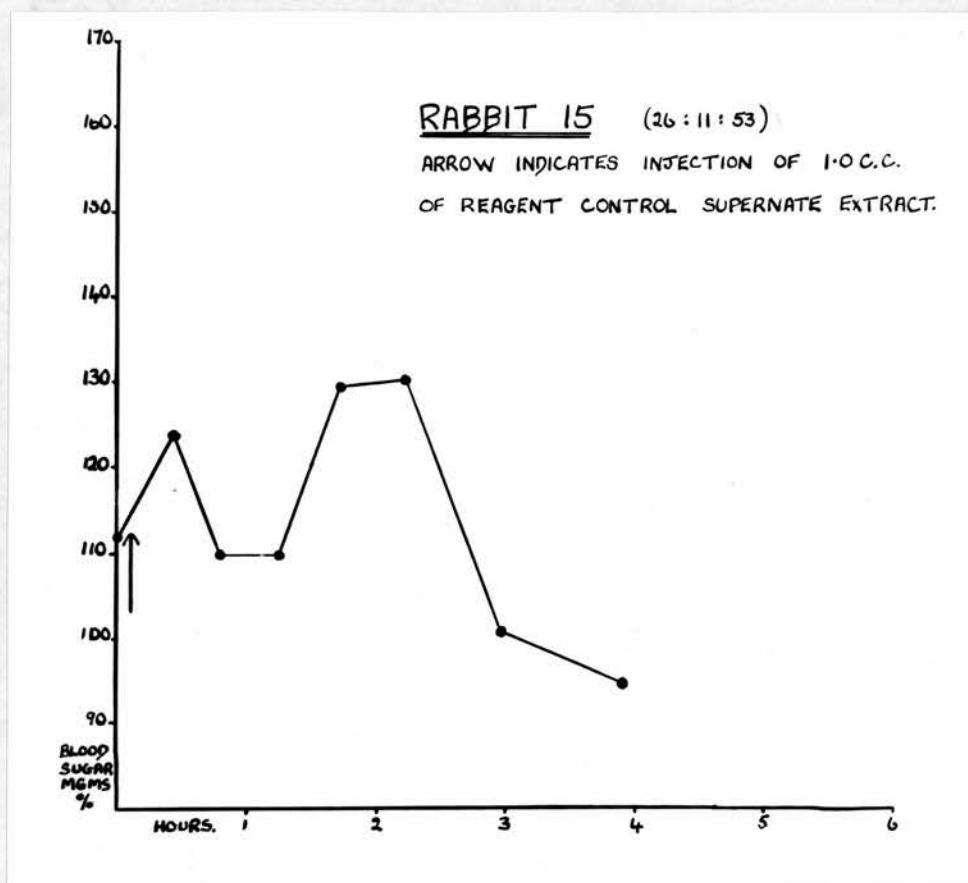
Measure the volume and precipitate the factor by adding 1.7 volumes of absolute alcohol and 2.8 volumes of ethyl ether. Leave overnight at 2°C. to ensure complete precipitation. Centrifuge and dry and precipitate at 2°C. in a desiccator.

The desiccated extracts of the 4 fluids were received back from Dr. Gilchrist as a fine dusting of white powder in the bottom of the tube. The extracts were suspended in each case in 2 m.l. of phosphate buffer (pH7.4) to which an equal volume of 0.9% NaCl. had been added. The resulting solutions, after centrifugation to remove insoluble material, were dialysed for 48 hours against chloride phosphate buffer (pH7.4) at 2°C. as recommended by Odée (1950). The supernate extract solutions were now frozen solid and stored at -25°C. until required for injection into the

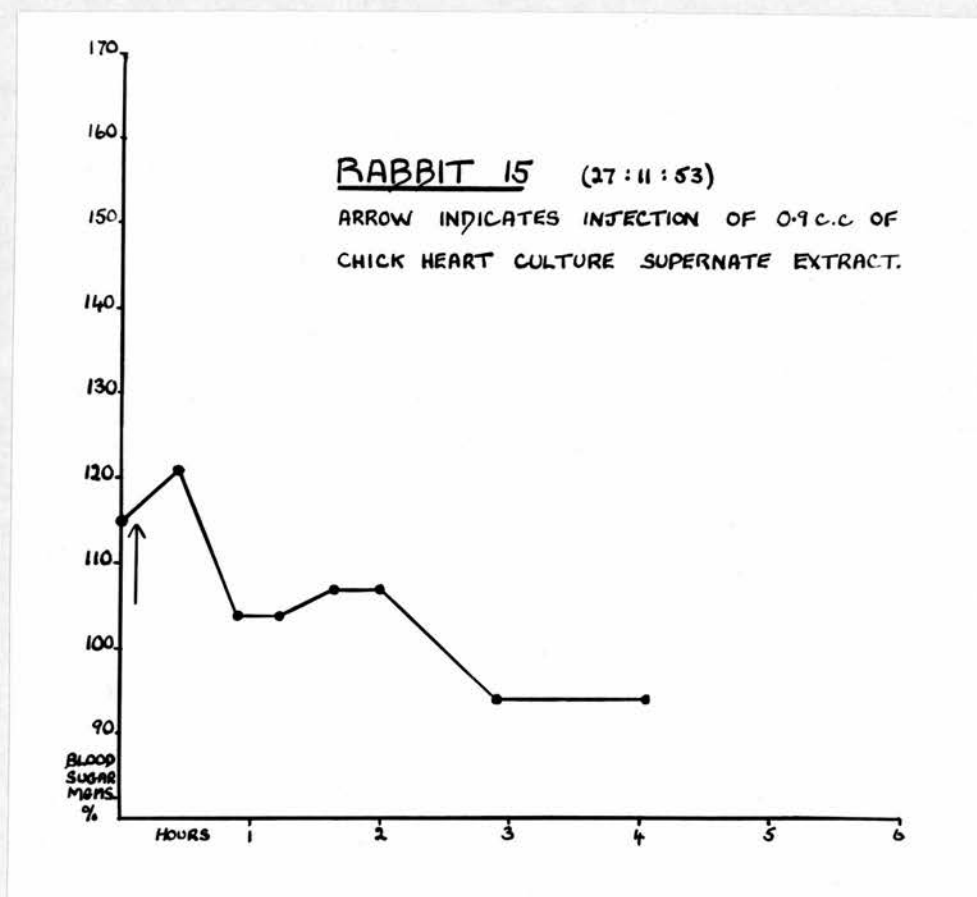
\* This was checked by titration.



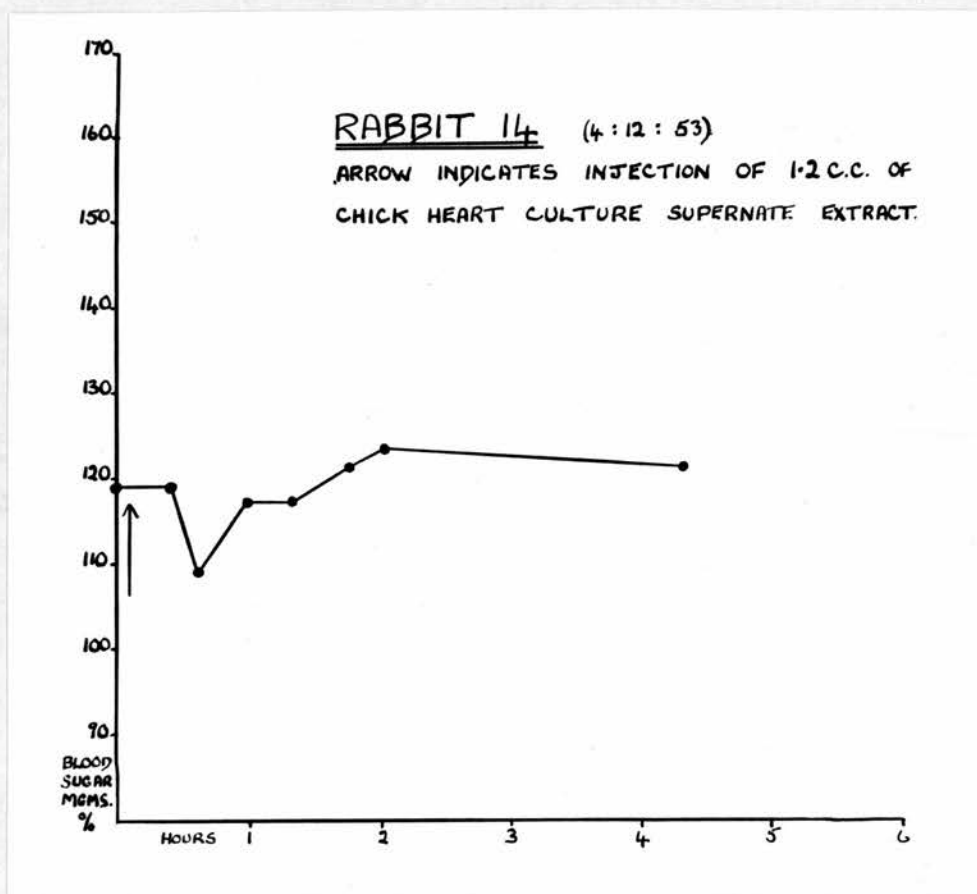
Graph 22.



Graph 23.

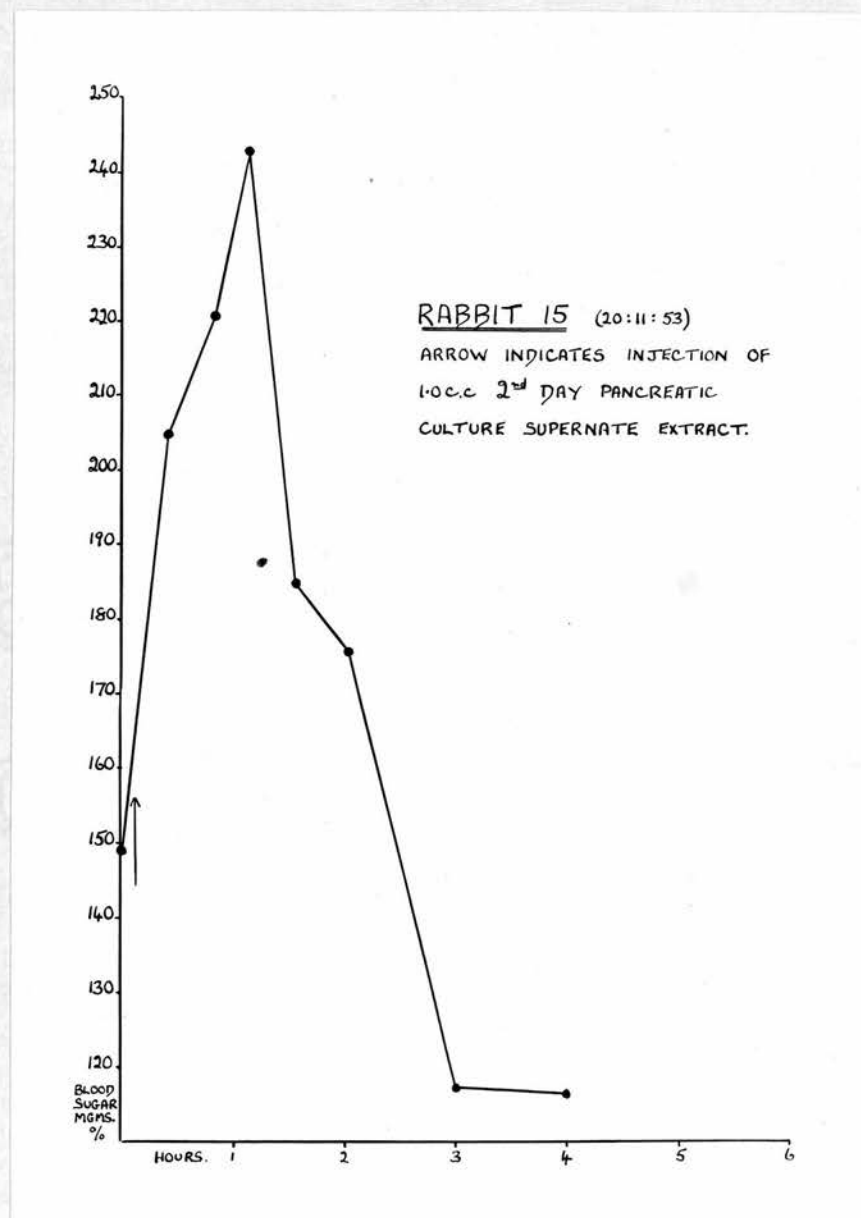


Graph 24.

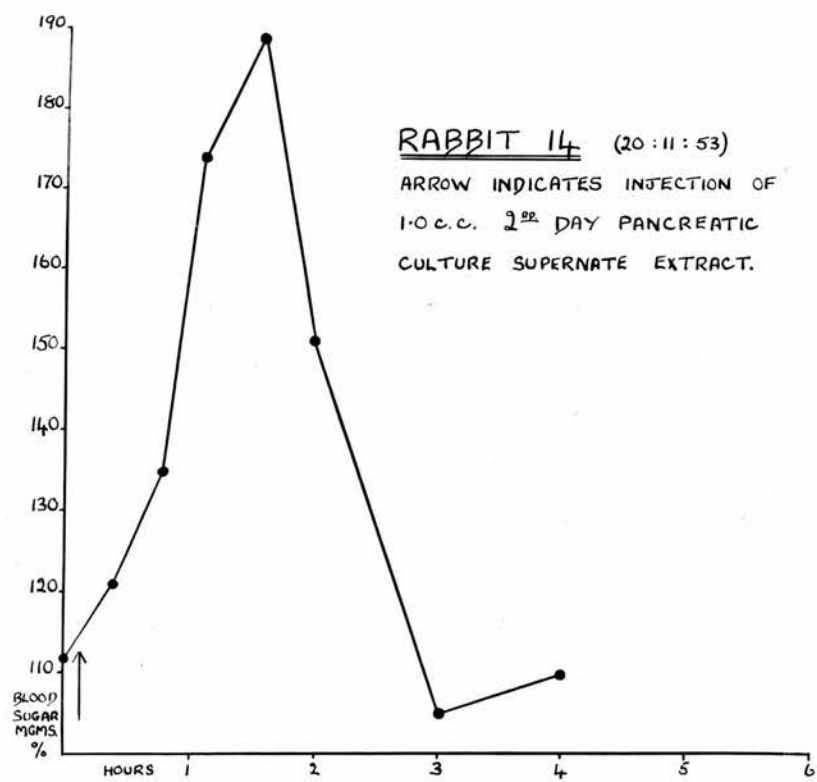


Graph 25.

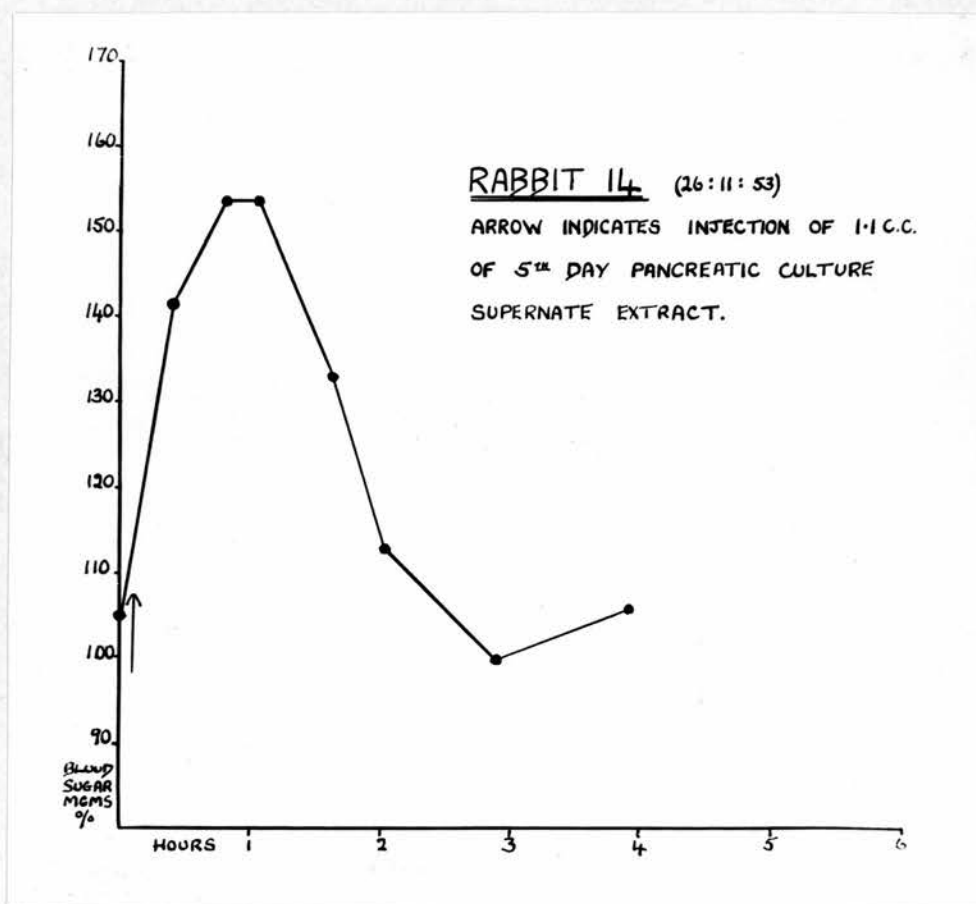




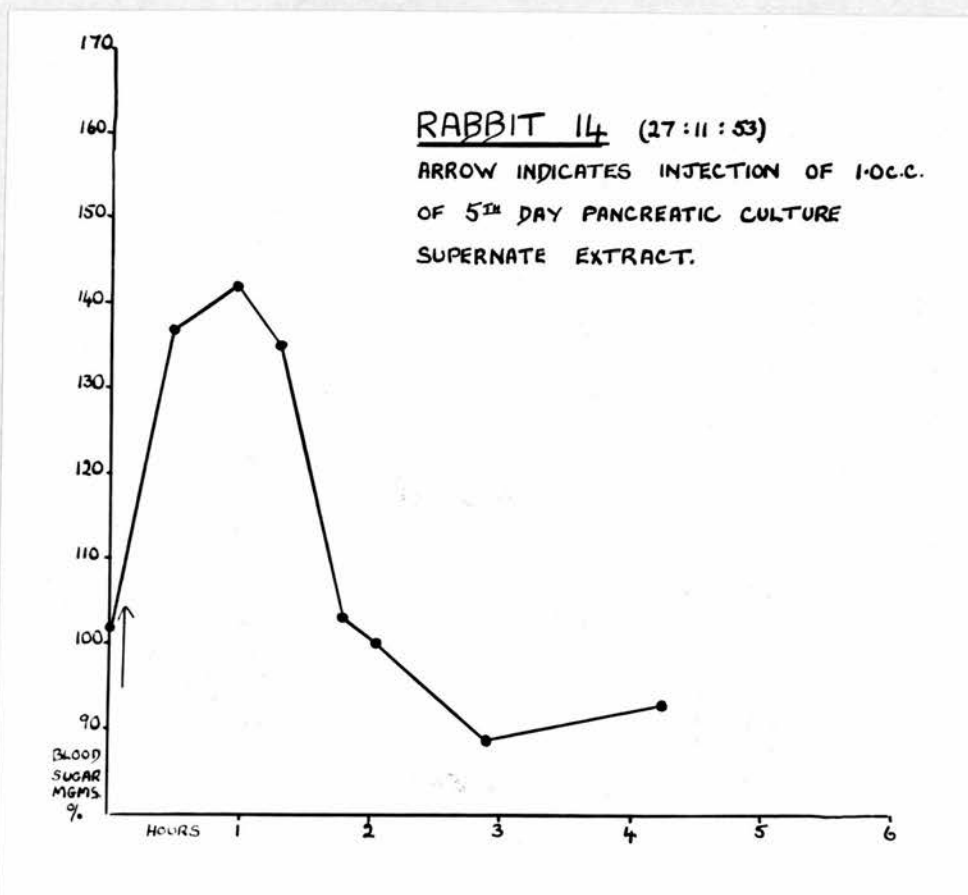
Graph 26.



Graph 27.



Graph 28.



Graph 29.

rabbits.

RESULTS OF INJECTION OF EXTRACTS.

- (1) Intravenous injections of the extract of pooled reagent control supernate were given on 2 occasions to Rabbit 15 and did not produce a significant hyperglycaemic curve. (c.f. Graphs 22 and 23).
- (2) Intravenous injections of the extracts of the pooled chick heart culture supernates were given to Rabbits 14, and 15 and produced no significant results. (c.f. Graphs 24 and 25).
- (3) Intravenous injections of the extract of the pooled pancreatic culture supernate taken on the second day of culture were given to Rabbits 14 and 15 and a very marked hyperglycaemic response resulted in one instance (c.f. Graph 26), and a more moderate, but very definite hyperglycaemic response was noted in Rabbit 14 (c.f. Graph 27).
- (4) Intravenous injections of the extract of the pooled pancreatic culture supernates from the fifth day of culture show a moderate hyperglycaemic response in both instances (c.f. Graphs 28 and 29).



Fig. 26.

Culture P.1. Haematoxylin and Eosin. x 65.  
 Explant consists chiefly of fibrous  
 granulation tissue. A rim of adherent  
 serous fluid can be seen at the edges.  
 (explanted 4 days).

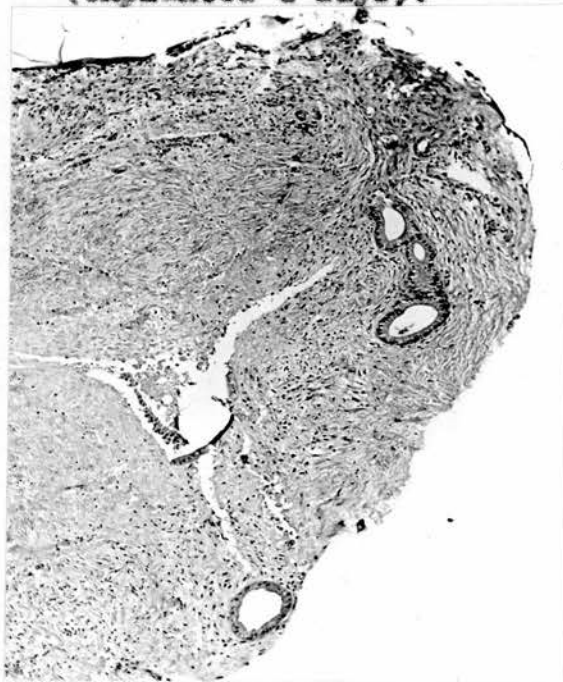


Fig. 27.

Culture P.4. Haematoxylin and Eosin. x 65.  
 Atrophic acini are seen and some dilated  
 pancreatic ductules. Central necrosis has  
 occurred in this explant.  
 (explanted 4 days).



A-cells.

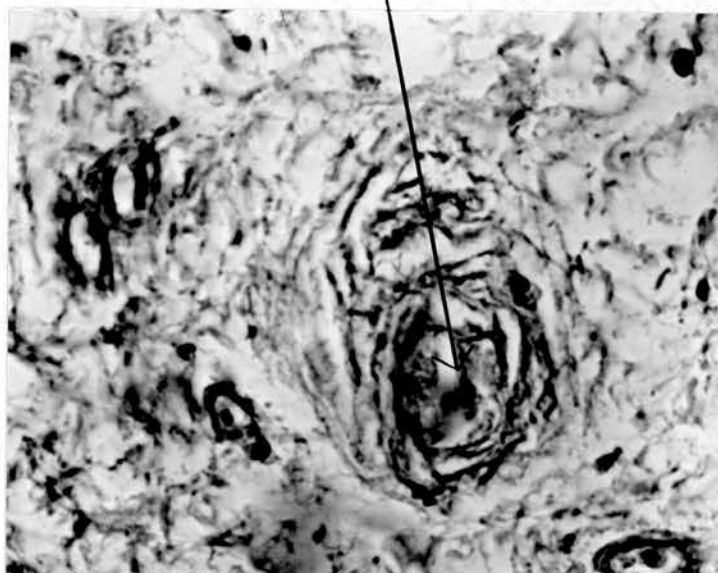


Fig. 28.

Culture P.5. Gomori. x 425. Red staining  
A-cells surrounded by fibrous bands.  
(explanted 4 days).

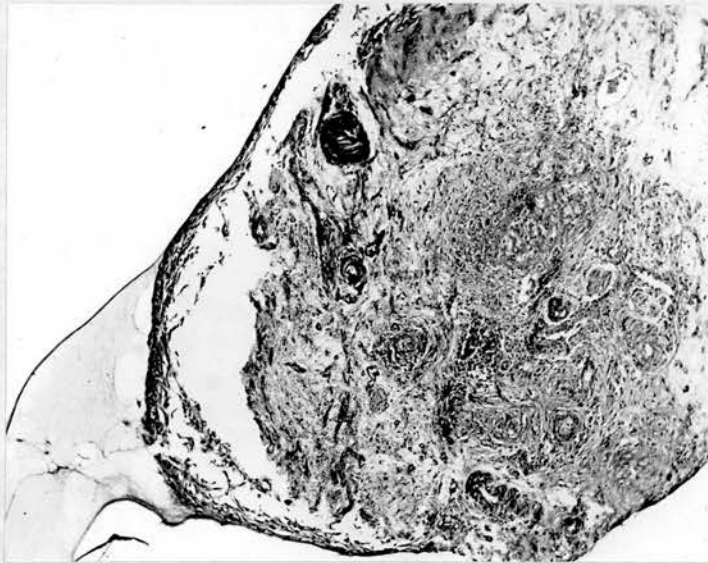


Fig. 29.

Culture P.11. Haematoxylin and Eosin. x 65.  
Groups of atrophic pancreatic acini  
surrounded by much fibrosis. Adherent  
clot is attached to the periphery of the  
explant. ( explanted 4 days).



Fig. 30.

Culture P.23. Haematoxylin and Eosin. x 65.  
Many groups of atrophic acini are seen  
surrounded by much fibrosis and showing a  
little fatty infiltration.  
(explanted 2 days)

## HISTOLOGY.

### i. Pancreatic explants.

The histological preparations of the explants showed that they consisted of fibrous tissue enclosing remnants of pancreatic acini and ductules. There was fairly widespread chronic inflammatory cell infiltration. Nerves and vessels were seen. Islets were identified in a number of explants. They consisted of red-staining A-cells with definite granules in many instances. These nests of A-cells were surrounded by fibrous bands. A very few of the explants examined showed central necrosis. Haemosiderin was found in many explants, resulting probably from haemorrhage produced at the time of ligation of the pancreas. (figs. 26 - 30).

### ii. Chick heart explants.

Heart cells and fibroblasts showed some growth, and a few showed areas of necrosis.

### iii. Ligated and unligated pancreas.

The ligated part of the pancreas of Dog 16 showed appearances similar to those seen in the explants. (fig. 31). Islets consisting of A-cells were identified. (fig. 32).

The unligated part of pancreas also shows



Fig. 31.

Dog 16 - sclerosed diabetic pancreas.  
Haematoxylin and Eosin. x 75. A main  
pancreatic duct surrounded by atrophic  
acinar tissue, fibrosis and round cell  
infiltration.

A-cells.

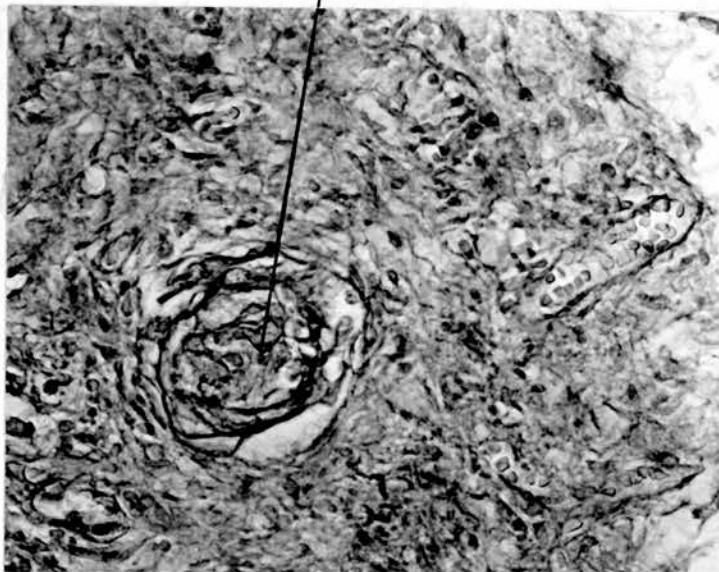


Fig. 32.

Dog 16. Sclerosed, diabetic pancreas.  
Gomori. x 425. A-cell islet invested  
by fibrous tissue.



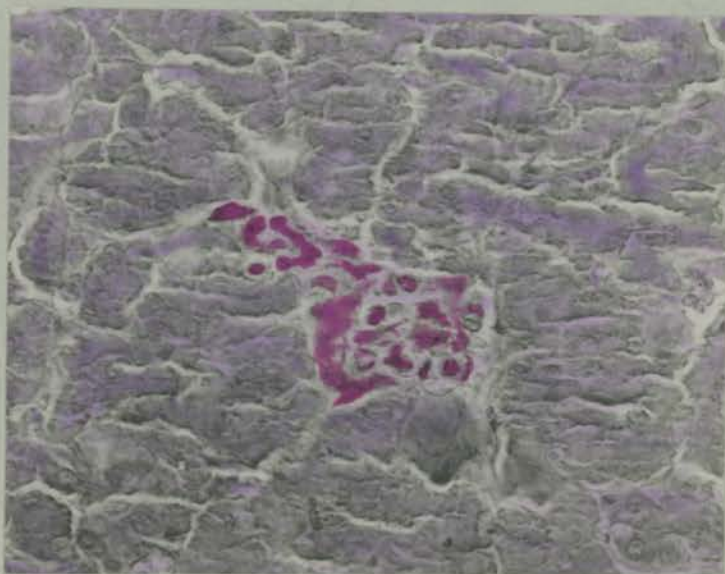


Fig. 33.

Dog 16. Unligated diabetic pancreas.  
Gomori. x 700. Islet consists mostly  
of A-cells. Tinted microphotograph.



many islets consisting entirely of A-cells.  
(fig. 33). (c.f. Appendix ii a. for description  
of pancreas).

Summary.

(Experiment 2)

- (1) An extract of the pooled reagent control supernates produces no sustained hyperglycaemic effect on rabbits.
- (2) An extract of the pooled chick heart culture supernates produces no sustained hyperglycaemic effect on rabbits.
- (3) An extract of pooled alloxan-diabetic, sclerosed pancreatic culture supernates of the second day of culture has a MARKED HYPERGLYCAEMIC effect on rabbits.
- (4) An extract of pooled alloxan-diabetic, sclerosed pancreatic culture supernates of the fifth day of culture has a MODERATE HYPERGLYCAEMIC effect on rabbits.
- (5) The histology confirms (a) that the explants consisted of sclerosed pancreas containing A-cell islets, and (b) that the ligated and unligated pancreas showed the characteristic changes of alloxan diabetes.

GENERAL DISCUSSION OF THE SIGNIFICANCE OF THE WORK  
IN SECTION III.

It may be seen from the summary at the end of experiment 1 that this experiment produced some results suggesting that in tissue culture, alloxan diabetic sclerosed dog pancreas might secrete a hyperglycaemic factor. These results were difficult to assess, as they were overlayed by the pronounced hyperglycaemic effect of the supernatant serum itself. In addition, due to the prolonged nature of the experiment, it was not possible to show in histological section the presence of healthy A-cell islets.

These deficiencies have been largely eliminated in the similar, but more refined experiment 2. In this experiment the time of culturing was short in order to obtain good histological preparations. The hyperglycaemic effect of serum had been eliminated by producing an extract free of serum proteins. Injection of this produced a sustained hyperglycaemic effect on rabbits only with the pancreatic explant supernate extracts and not in the case of the reagent control extract or in the case of the heart explant supernate extract.. The hyperglycaemic effect of the extracts from the pancreatic explant supernates was less pronounced with the extracts of fifth day supernates than with the second day supernate

extracts.

Thus, this latter work suggests that there is present in the supernatant fluids of alloxan diabetic, sclerosed pancreatic explants in Carrel flasks, a hyperglycaemic substance which is probably present in a higher concentration early in the experiment, but which is still present even after the first supernatant fluid has been removed and replaced. It seems, therefore, likely, that it is secreted by the cultures and probably by the A-cells as these are the only functional and potentially secretory cells remaining in any number in the sclerosed diabetic pancreas.

These observations are supported by the histological evidence of healthy A-cell islets in the explants and in sections of the ligated and unligated pancreas from which the explants are derived. These results should be considered in the light of the statement of Sutherland and de Duve (1948) that their glycogenolytic factor had not yet been proved to be a second pancreatic hormone, but "that the best proof would be to show that it was secreted into the blood stream and participated in the regulation of the blood sugar level." As discussed in the Introduction to this thesis, this proof has been attempted by Foa et alii (1949) and by Cavallero and Malandra (1950 a & b). It is thought that the present work adds to the

findings of these authors in that it illustrates the presence of a hyperglycaemic factor in the supernatant fluid of a culture of alloxan-diabetic sclerosed pancreas. As this tissue in fact contains only A-cells, connective tissue and histiocytes, it would be reasonable to suppose that this hyperglycaemic factor was secreted by the A-cells. This secretion is of a nature analogous to true endocrine secretion and there is no exocrine part of the pancreas functional, as there was in the cross circulation experiments of Foa et alii (1949) and in the pancreatic grafts of Cavallero and Malandra (1950 a & b).

It has unfortunately not been possible to repeat this work yet. As can be understood by reference to Section II, there is difficulty in preparing the necessary animal from which to obtain the alloxan diabetic sclerosed pancreas. At the moment, however, attempts are being made to prepare more such dogs with a view to:

- i. repeating the type of investigation here described in experiment 2.
- ii. undertaking further experiments in which cobalt chloride and Synthalin A, (c.f. Van Campenhout and Cornelis, 1951 a & b. and Davis, 1952), are administered to destroy the A-cells. If, after the administration of these substances, hyperglycaemic secretion can now be eliminated, it would

further confirm that the hyperglycaemic factor does in fact originate from the pancreatic A-cells and is a second pancreatic hormone, as seems to be indicated by the previous work reviewed in the Introduction, and by the work now recounted.

Summary.

- (1) 2 groups of experiments have been described in which alloxan diabetic, sclerosed dog pancreas was explanted into a biphasic medium in Carrel flasks.
- (2) Intravenous injections of supernates and serum free extracts of supernates from the flasks produce a significant, sustained hyperglycaemia in rabbits.
- (3) These results are adduced as further evidence suggesting that the pancreatic A-cells secrete a hyperglycaemic substance which may be a second pancreatic hormone.

SECTION IV.



ATTEMPTED PREPARATION OF ALLOXAN DIABETIC  
HYPOPHYSECTOMIZED, ADRENALECTOMIZED RATS  
(A.D.H.A. RATS).

Experiment (1)

19.7.52

The method followed was in the main that of Bornstein (1950).

24 adult male rats of Glaxo strain were used. The weight recommended by Bornstein is 220-240 G., but as this particular weight was not obtainable at the time, the weights tended to be a little lower as can be seen by reference to Table (1), though generally they were in the 170-215 G. range. The rats were identified by ear-marks.

I. Injection of alloxan.

A 5% solution of alloxan was made up in distilled water. The rats were given a dose equivalent to 50 mgm. alloxan/Kg. of body weight. (In all the experiments here described the alloxan was given between 3 and 6 p.m.).

The animals without any particular preparation were anaesthetized with ether. The (R) jugular vein was exposed and the alloxan

injected intravenously from a 1 c.c. tuberculin syringe. The incision was closed with a single suture.

As may be seen on the Table, 3 rats died within 72 hours of injection. 36 hours after injection, urine was collected from all the rats. This was done by lifting the animals on to a porcelain plate when the majority would urinate. Others required to be partially anaesthetized with ether and would then pass water. No failures were experienced by these methods. The urine was drawn from the plate by a fine Pasteur pipette and 5 drops placed in a Kahn tube to which 2.5 c.c. of Benedict's solution were added. In a small number of cases only 2 drops of urine could be obtained. Using correspondingly less Benedict's solution this appeared to give satisfactory results. The results of the Benedict's test for glycosuria were recorded thus -

Slight turbidity	= trace (tr.)
Definite yellow precipitate	= +
Orange precipitate	= ++
Brick red precipitate	= +++

It was found 6 rats showed no glycosuria. At 72 hours after injection, the urine was again tested for sugar in those rats which were negative at 36 hours. 1 rat had become positive (++) and 1 had died.

From 36 hours - 96 hours 4 rats which

appeared lethargic and drowsy were given 4 units of zinc protamine insulin subcutaneously each day. No insulin was given after 96 hours. 1 such drowsy rat died. Those rats having (+++) urinary sugar were not the ones appearing drowsy. It should be noted that these indications for insulin administration are not the same as those of Bornstein (1950) who accepted heavy glycosuria as an indication for insulin administration. The dose of insulin, however, is that recommended by Bornstein. No insulin was given from the 4th - 6th day to avoid the risk of hypoglycaemic attacks (Bornstein, 1950).

## II. Hypophysectomy (referred to as hypox. in the Tables).

On the 6th day after the administration of the alloxan the remaining rats (17) were given 0.25 G. glucose intra-peritoneally in 5% saline after anaesthetising with ether, with the object of preventing hypoglycaemia as recommended by Bornstein (1950).

The hypophysectomy (Dr. Taylor) was performed by the parapharyngeal approach, using a dental drill.

During induction of anaesthesia 2 rats died (both (+++) glycosuria). In several others, anaesthesia caused collapse from which they were

retrieved with difficulty. During hypophysectomy no deaths occurred, but within the following 24 hours 5 more deaths took place.

48 hours after hypophysectomy urinary sugars were performed on the survivors (10) and only 1 was positive (+) indicating the adequacy of the hypophysectomy which had thus cured or alleviated the diabetes.

### III. Adrenalectomy.

Adrenalectomy was performed 16 days after hypophysectomy, by which time 2 more rats had died.

Method: Ether anaesthesia - Funnel. A single midline incision through skin and superficial fascia of the back extending from the level of the last rib 2 c.m. caudally. A muscle incision 3 m.m. long was made through the abdominal muscle at the border of sacro-spinalis. The muscle was retracted and the dorsum of kidney exposed. Above and internal to the kidney the suprarenal lay, usually surrounded by fat. Traction on the latter brought the suprarenal to view and it was grasped from below with a pair of curved fine forceps and shelled out in one piece. Usually little or no bleeding resulted from this procedure. The muscle was not stitched except in one instance where the suprarenal was difficult to find and the incision had to be enlarged. The skin incision

was sutured. 1 rat died in inducing anaesthesia and 1 bled severely from a perinephric vein.

The 7 surviving rats were put in wire cages, with wooden floors covered in sawdust. Food pellets and saline were constantly available. The temperature of the room was 22°C. All the rats except 1 died within 2 - 7 days following adrenalectomy, although salt was available in the drinking water.

Rat 7 survived for many weeks before succumbing also. At this time it was not convenient to repeat this work, and the attempts to prepare A.D.H.A. rats were not renewed until November, 1953.



19.7.52.

No. of rat	Weight G.	Urinary Sugars (Benedict)				Comments
		36 hrs. after alloxan	72 hrs. after alloxan	48 hrs. after hypox.	48 hrs. after adrenalectomy	
1	215	++		NEG.	XXXXXXXXXXXX	Died during adrenalectomy.
2	215	NEG.	NEG.			Failed alloxan diabetes.
3	170	++		NEG.	XXXXXXXXXXXX	Died after adrenalectomy.
4	214	+++			XXXXXXXXXXXXXXXXXXXX	Died during hypox.
5	137	NEG.	NEG.			Failed alloxan diabetes.
6	140	++		NEG.	XXXXXXXXXXXX	Died after hypox. (4 days).
7	203	++		NEG.		SURVIVED SEVERAL WEEKS.
8	214	+			XXXXXXXXXXXXXXXXXXXX	Died after hypox.
9	145	NEG.			XXXXXXXXXXXXXXXXXXXX	Died after alloxan.
10	214	NEG.	NEG.			Failed alloxan diabetes.
11	214	++			XXXXXXXXXXXXXXXXXXXX	Died after alloxan.
12	214	+		NEG.	XXXXXXXXXXXX	Died after adrenalectomy.
13	214	++			XXXXXXXXXXXXXXXXXXXX	Died after hypox.
14	210	++		NEG.	XXXXXXXXXXXX	Died after adrenalectomy.
15	215	+			XXXXXXXXXXXXXXXXXXXX	Died after hypox.
16	170	NEG.	++		XXXXXXXXXXXXXXXXXXXX	Died after hypox.
17	178	++		NEG.	XXXXXXXXXXXX	Died after adrenalectomy.
18	170	++			XXXXXXXXXXXXXXXXXXXX	Died after alloxan.
19	214	++		+	XXXXXXXXXXXX	Died after adrenalectomy.
20	140	+++			XXXXXXXXXXXXXXXXXXXX	Died during hypox.
21	175	++			XXXXXXXXXXXXXXXXXXXX	Died after hypox.
22	145	++		NEG.	XXXXXXXXXXXX	Died after adrenalectomy.
23	140	++		NEG.	XXXXXXXXXXXX	Died 10 days after hypox.
24	188	NEG.	NEG.			Failed alloxan diabetes.

XXXXXXXXXX means animal is dead.



Experiment(2)1.11.53

Even though a survival rate of significant proportions was not obtained in July 1952 and indeed, because of it, it was determined to investigate the method thoroughly with a view to determining -

- (1) Whether the method of preparation was valid;
- (2) Whether the experiments of Bornstein, Reid and Young (1951) with growth hormone in A.D.H.A. rats could be confirmed;
- (3) Whether the injection of growth hormone into an A.D.H.A. rat was as effective in causing secretion of H.G.F. (glucagon) into the portal blood after pancreatectomy as before it; in short, to investigate further whether growth hormone was acting on the pancreas to stimulate production of glucagon.

30 rats of the Glaxo strain were used. The weights varied between 70 and 275 G. Most were approximately 150 G. They were taken as available without regard to sex. Alloxan 50 mgm./Kg. of body weight was given into the jugular vein. 1 rat had died by the next day.

At 36 hours from commencing the experiment urine was taken from all the rats and examined for sugar by Benedict's test. The results may be seen in Table (2).

No insulin was given to these rats with the object of determining whether insulin was necessary, and by omitting it perhaps reducing the mortality from hypoglycaemia during and after hypophysectomy. On the third day, almost all the rats were dead or dying. A random 6 were autopsied and no naked eye lesions were found.

On the fourth day only 3 rats were left alive and by the ninth day only 2 remained. It was decided not to hypophysectomize these at that time, but to retain them for hypophysectomy with a larger group which might be obtained in subsequent attempts.

It is concluded, therefore, that insulin should be given to prevent death from diabetic coma following the administration of 50 mgm. alloxan/Kg. of body weight.

Reference to the microscopic reports on the rats autopsied shows that 2 died as a result of diabetes mainly, 2 as a result of renal tubular damage mainly, and 2 from a combination of diabetes and tubular damage. Insulin might have saved the diabetic animals and possibly tided over some others which had diabetes and kidney damage until they recovered from their renal lesions (c.f. Appendix ii b.).

TABLE (2). PREPARATION OF A.D.H.A. RATS. EXPERIMENT 2.  
1.11.53.

No. of rat	Weight G.	Urinary Sugars (Benedict)				Comments
		36 hrs. after alloxan	72 hrs. after alloxan	48 hrs. after hypox.	48 hrs. after adrenalectomy	
25	275	NEG.	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
26	145	+++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
27	140	+++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
28	125	+		XXXXXXXXXXXXXXXXXXXXXXXXXXXX		Died after hypox.
29	75	+	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
30	140	++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
31	140	+++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
32	150	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
33	205	+++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
34	150	+	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
35	145	++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
36	125	+++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
37	70	NEG.	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
38	90	NEG.	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
39	160	+	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
40	150	NEG.	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
41	160	+	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
42	145	++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
43	140	+++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
44	110	+++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
45	125	tr.	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
46	100	+++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
47	100	+++		XXXXXXXXXXXXXXXXXXXXXXXXXXXX		Died after hypox.
48	140	+	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
49	95	tr.	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
50	95	+	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
51	150	++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
52	125	+++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
53	135	+	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.
54	145	+	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	Died after alloxan.

XXXXXXXXXX means animal is dead.

Experiment (3)15.11.53

A further attempt to prepare this animal was undertaken.

24 rats (Glaxo) were used. They were taken irrespective of sex. Weights varied from 105 - 205 G., but most were 125 - 150 G.

Alloxan was given by the same technique as in Experiments (1) and (2). Urine was examined for sugar at 36 hours after injection. 48 hours after alloxan was given, all rats with a positive urinary sugar had 4 units zinc protamine insulin subcutaneously in the hope of preventing the very large number of deaths occurring after alloxan in Experiment (2) where no insulin was used. It appeared to achieve some improvement for only 11 rats died, but 3 of these were having convulsions presumably due to hypoglycaemia produced by the insulin. They were all rather light animals (115, 125, 125 G.). 5 of the rats were autopsied and 3 showed severe islet damage and no kidney lesions and had died of diabetes, 2 showed islet damage, no kidney damage and had died with convulsions due to insulin. All rats with a negative urinary sugar at 36 hours were examined again at 72 hours as in previous experiments. Those still negative (3) were discarded.

10 rats + 2 diabetic rats from Experiment (2) were sent to hypophysectomy (Dr. Loraine). 4 returned with obvious brain damage and hemiplegia and died. 1 died during anaesthesia and the remainder died in the 2 or 3 days following hypophysectomy. Post-operatively all the shocked rats had intra-peritoneal injections of 5% glucose saline to combat shock, chloride deficiency and possible hypoglycaemia. The rats were kept warm at 23°C. after hypophysectomy.

It is therefore concluded that it is inadvisable to give all diabetic rats insulin, and that it should be reserved for the drowsy ones seen 48 hours after alloxan. The mortality of hypophysectomy in diabetic rats seems to be very high, but it is planned to try this experiment once more.



15.11.53.

No. of rat	Weight G.	Urinary Sugars (Benedict)				Comments
		36 hrs. after alloxan	72 hrs. after alloxan	48 hrs. after hypox.	48 hrs. after adrenalectomy	
55	105	+++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX			Died after alloxan.
56	130	++		XXXXXXXXXXXXXXXXXXXXXXXXXXXX		Died after hypox.
47	-	-	-	-	-	Recorded with Experiment 2.
57	125	+		XXXXXXXXXXXXXXXXXXXXXXXXXXXX		Died after hypox.
58	135	++		XXXXXXXXXXXXXXXXXXXXXXXXXXXX		Died after hypox.
59	125	+++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX			Died after alloxan (insulin convulsion)
60	125	++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX			Died after alloxan.
61	135	++		XXXXXXXXXXXXXXXXXXXXXXXXXXXX		Died after hypox.
28	-	-	-	-	-	Recorded with Experiment 2.
62	205	++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX			Died after alloxan.
63	145	++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX			Died after alloxan.
64	140	+	XXXXXXXXXXXXXXXXXXXXXXXXXXXX			Died after alloxan.
65	130	tr.	NEG.			Failed to become diabetic.
66	155	+		XXXXXXXXXXXXXXXXXXXXXXXXXXXX		Died after hypox.
67	125	++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX			Died after alloxan (insulin convulsion)
68	135	+	XXXXXXXXXXXXXXXXXXXXXXXXXXXX			Died after alloxan.
69	130	+		XXXXXXXXXXXXXXXXXXXXXXXXXXXX		Died after hypox.
70	130	+		XXXXXXXXXXXXXXXXXXXXXXXXXXXX		Died after hypox.
71	115	++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX			Died after alloxan (insulin convulsion)
72	115	tr.	XXXXXXXXXXXXXXXXXXXXXXXXXXXX			Died after alloxan.
73	150	tr.	NEG.			Failed to become diabetic.
74	145	+		XXXXXXXXXXXXXXXXXXXXXXXXXXXX		Died after hypox.
75	125	+++	XXXXXXXXXXXXXXXXXXXXXXXXXXXX			Died after alloxan.
76	130	tr.	NEG.			Failed to become diabetic.
77	125	++		XXXXXXXXXXXXXXXXXXXXXXXXXXXX		Died during hypox.
78	120	+		XXXXXXXXXXXXXXXXXXXXXXXXXXXX		Died after hypox.

XXXXXXXXXX means animal is dead.



Experiment (4)29.7.54.

In this attempt 29 rats of the Glaxo strain were chosen regardless of sex. They were selected to be of rather low weight, mostly of about 50 G., but varying between 25 G. and 90 G. This was done because

- (1) it was felt that a milder type of diabetes might develop in a young rat, which in fact, proved to be the case. It was hoped thus to increase the survival rate;
- (2) the hypophysectomist (Dr. Loraine) considered smaller diabetic rats would survive hypophysectomy more readily.

Of the 29 rats given 50 mgm. alloxan/Kg. of body weight, 16 became diabetic. (Sutures were not used on this occasion for closing the neck incision after injection, as they were usually bitten out, and the wounds healed naturally with remarkable rapidity).

It was intended to give any drowsy rat insulin, but none became drowsy. This suggests that young rats may be less susceptible to alloxan than older ones, due possibly to regeneration of islets from ductules (c.f. Appendix ii b. Rat <sup>81</sup>85).

As can be seen on Table (4), 16 rats went

to hypophysectomy. They had been drinking 0.9% N. saline instead of water for 48 hours previously. Hypophysectomy was performed 8 days after alloxan had been given. Dr. Loraine injected a few of the rats done first with the glucose-saline solution recommended by Bornstein, but was forced to the opinion that they were doing better without it.

The rats were treated for shock immediately after hypophysectomy by warming near a radiator, and the saline was now given in 5% glucose for 2 days. After 2 days they continued on 0.9% N. saline to drink and rat pellets as food. 5 rats survived the immediate post-operative 48 hours. These animals were found to have reverted now to a NEGATIVE urinary sugar. 1 died 6 days after hypophysectomy.

Bilateral adrenalectomy technique. 14.12.53.

6 intact rats (weights 120 - 160 G.) were subjected to bilateral adrenalectomy having had 0.9% N.Na.Cl. substituted for water 24 hours previous to operation.

Technique: Animals were anaesthetized in a jar of ether and laid ventral surface downwards on cork mats. A midline skin incision 3 c.m. long was made posteriorly. The muscle was divided at the lateral border of sacro-spinalis immediately inferior to the rib margin. The incision was 1 c.m.

long. The kidney was then seen. Fine forceps were used to grip the peri-adrenal fat. The adrenal was then gripped by curved 'mosquito' forceps placed below until it became congested. It was then enucleated with dissecting forceps. This technique avoids any haemorrhage as the adrenal vessels are crushed by the 'mosquito' forceps. The same procedure was repeated on the other side through a separate muscle incision, but the same skin incision. A single suture was put in the skin and in each muscle incision.

2 c.c. of 5% saline is then injected subcutaneously. The rats are then kept warm to minimise shock. They had 0.9% N.Na.Cl. in 5% glucose for 48 hours and then 0.9% N.Na.Cl. alone + rat pellets. They all survived for 2 - 3 weeks.

The 4 rats surviving hypophysectomy were prepared for bilateral adrenalectomy on the 10th day after hypophysectomy thus:- 24 hours before operation, their wounds having now healed, they were given 1.0 mgm. desoxycorticosterone acetate (D.O.C.A.) intramuscularly, and were put on 0.9% N. saline for 48 hours before operation.

They had bilateral adrenalectomy performed by the above technique. The operations were all technically highly satisfactory. 2 c.c. 5% saline was given subcutaneously during operation. The rats were then put on 5% glucose saline in their

water bottles. 3 were dead the following morning.  
The other died 3 days later.

TABLE (4).

PREPARATION OF A.D.H.A. RATS.

EXPERIMENT 4.

29.11.53.

No. of rat	Weight G.	Urinary Sugars (Benedict)				Comments.
		36 hrs. after alloxan	72 hrs. after alloxan	48 hrs. after hypox.	48 hrs. after adrenalectomy	
79	90	+++		NEG.	XXXXXXXXXXXX	Died after adrenalectomy.
80	40	NEG.	NEG.			Failed to become diabetic.
81	60	++		XXXXXXXXXXXXXXXXXXXX		Died after hypox.
82	95	+		XXXXXXXXXXXXXXXXXXXX		Died after hypox.
83	75	NEG.	NEG.			Failed to become diabetic.
84	75	XXXXXXXXXXXXXXXXXXXX		XXXXXXXXXXXXXXXXXXXX		Died 24 hrs. after alloxan.
85	45	+		XXXXXXXXXXXXXXXXXXXX		Died during hypox.
86	60	+++		NEG.	XXXXXXXXXXXX	Died 3 days after adrenalectomy.
87	60	++		XXXXXXXXXXXXXXXXXXXX		Died after hypox.
88	45	tr.	tr.			Failed to become diabetic.
89	50	++		XXXXXXXXXXXXXXXXXXXX		Died during hypox.
90	60	+		NEG.	XXXXXXXXXXXX	Died 6 days after hypox.
91	25	XXXXXXXXXXXXXXXXXXXX		XXXXXXXXXXXXXXXXXXXX		Died 24 hrs. after alloxan.
92	50	XXXXXXXXXXXXXXXXXXXX		XXXXXXXXXXXXXXXXXXXX		Died 24 hrs. after alloxan.
93	80	+++		XXXXXXXXXXXXXXXXXXXX		Died after hypox.
94	70	tr.	NEG.			Failed to become diabetic.
95	55	NEG.	NEG.			Failed to become diabetic.
96	30	XXXXXXXXXXXXXXXXXXXX		XXXXXXXXXXXXXXXXXXXX		Died 24 hrs. after alloxan.
97	35	+++		XXXXXXXXXXXXXXXXXXXX		Died during hypox.
98	75	++		XXXXXXXXXXXXXXXXXXXX		Died after hypox.
99	30	+++		XXXXXXXXXXXXXXXXXXXX		Died after hypox.
100	70	XXXXXXXXXXXXXXXXXXXX		XXXXXXXXXXXXXXXXXXXX		Died 24 hrs. after alloxan.
101	40	+		NEG.	XXXXXXXXXXXX	Died after adrenalectomy.
102	50	NEG.	NEG.			Failed to become diabetic.
103	40	NEG.	NEG.			Failed to become diabetic.
104	60	+		XXXXXXXXXXXXXXXXXXXX		Died after hypox.
105	75	++		NEG.	XXXXXXXXXXXX	Died after adrenalectomy.
106	50	+++		XXXXXXXXXXXXXXXXXXXX		Died after hypox.
107	50	NEG.	NEG.			Failed to become diabetic.

XXXXXXXXXX means animal is dead.



### Discussion.

It is not proposed to repeat this (A.D.H.A.) preparation, because the method is considered highly unlikely to produce a significant number of survivors. Firstly at best one can hope for approximately 50 - 60% of the rats to become diabetic and stay alive.

When these are subjected to hypophysectomy the number of survivors is variable, but usually very small even in the hands of an experienced operator (e.g. Dr. Loraine normally has approximately 80% survival on intact rats of weight range 40 - 60 G.).

Of the very few surviving hypophysectomy, all or almost all, succumb to adrenalectomy even when this is done with technical competence, and when apparently adequate pre- and post-operative precautions were taken, i.e. precautions quite adequate to secure survival in intact rats subjected to bilateral adrenalectomy. Therefore, as even with some experience of the method a heavy mortality occurs at each stage NO significant survival rate can be expected. It may be noted that attempts to prepare this A.D.H.A. rat by the method of Bornstein have also been made in Toronto and have likewise met with failure (Best, 1953).

It has been suggested that a higher



survival rate might be obtained by removing the pituitary first and thus cutting down the very great risk inherent in subjecting diabetic rats to hypophysectomy (Salter, 1953). A high success rate is claimed by this method. The objections to it are, however, readily understood.

(1) Hypophysectomy done first means that rats which have sustained significant B-cell damage will not show glycosuria;

(2) It is a more usual finding that alloxan will produce diabetes in only 50% of rats and not in 100% as Salter (1953) found. (Gaarenstroom, 1946). That has been the experience of this investigation also.

(3) Bearing these facts in mind, it is evident that of the rats surviving hypophysectomy and given alloxan - only half will have sustained significant damage to the B-cells. Some will have died of the alloxan and approximately a quarter will survive, but will probably not be diabetic. So the number of rats which survive the whole procedure will contain a very significant number of hypophysectomized adrenalectomized rats which never were diabetic and many truly diabetic rats will have died. So one may conjecture that of the hypophysectomized rats given alloxan 50% will be diabetic, 25% at least will die, and 25% will not be diabetic. Again, the non-diabetic hypophysectomized rats subjected to adrenalectomy will tend to survive more readily

than the diabetic ones, so that in the final count perhaps half the survivors are truly A.D.H.A. rats. The remainder are hypophysectomized, adrenalectomized rats.

(4). Further, if urinary sugars are not done before and after hypophysectomy in order to observe the reversion to a negative urinary sugar which occurs in a diabetic rat when the pituitary diabetogenic factor is no longer secreted, then there is no method of knowing whether the hypophysectomy was complete or not until death and subsequent histological examination.

It is therefore concluded that performing the hypophysectomy first would not solve the problem of preparing these A.D.H.A. rats. Some of the animals prepared by this latter method might be suitable for insulin assay, but certainly not for repeating an experiment of the type reported by Bornstein, Reid and Young (1951). Such rats would clearly be unsuitable also for the growth hormone - pancreatectomy experiment which was outlined in the Introduction to this thesis.

It was observed in Experiment 4 that younger rats seemed to succumb less readily to the diabetogenic action of alloxan. In such rats regeneration of islets from ductules was demonstrated. (figs. 65 and 66.).

Summary

- (1) 4 attempts have been made to prepare alloxan diabetic hypophysectomized rats, originally following the method of Bornstein (1950) and subsequently modifying it in the hope of securing more survivors.
- (2) All these attempts failed to produce a significant number of survivors.
- (3) The modification in the preparation used by Salter (1953) is discussed.
- (4) The proposal to repeat the experiments of Bornstein, Reid and Young (1951) and to carry out the growth hormone-pancreatectomy experiment outlined in the Introduction is accordingly abandoned as the necessary animals cannot be prepared.
- (5) It was noted that younger rats of lighter weight do not appear to succumb so readily to the diabetogenic action of alloxan, possibly due to islet regeneration from ductules.

SECTION V.

SUMMARY AND CONCLUSIONS.

As indicated in the Introduction to this thesis, the results of the experimental work have been discussed and summarized at the end of the appropriate section. Therefore, the summary here set down is brief to avoid unnecessary repetition, and only points relevant to the main argument of the thesis are mentioned. Other observations of interest are recorded in the Section summaries and in the appendices.

Previous Work.

The literature relevant to glucagon and its possible secretion by the pancreatic A-cells has been fully discussed in Section I. It will be recalled that Sutherland and de Duve (1948) referring to the hyperglycaemic, glycogenolytic factor which they had extracted from alloxan diabetic, sclerosed dog pancreas were reluctant to assume that it was a second pancreatic hormone "in the absence of a clear cut demonstration that it is secreted into the blood stream, and that it participates in the regulation of the blood sugar level." (c.f. P.13).

A number of attempts have been made to demonstrate such a secretion into the blood stream.

Foa et alii (1949), in cross circulation

experiments in dogs, found that an alloxan diabetic dog apparently secreted a hyperglycaemic substance into its pancreatico-duodenal vein. In these experiments, however, the exocrine tissue of the pancreas was still functional, and it could be alleged that the hyperglycaemic substance derived from that. (c.f. P.16).

Cavallero and Malandra (1950 a. and b) using pancreatic grafts placed subcutaneously and intraperitoneally in rats, thought that a hyperglycaemic substance was secreted by the pancreas, and that it probably came from the A-cells. Their results, however, were not entirely conclusive, and again exocrine pancreatic tissue had not been eliminated. (c.f. P.17).

Bornstein, Reid and Young(1951) reported a most interesting experiment in which pituitary growth hormone was given to alloxan diabetic, hypophysectomized, adrenalectomized (A.D.H.A.) rats. The portal blood of these animals, after such an injection, was found to contain a hyperglycaemic substance when assayed on another A.D.H.A. rat. These experiments strongly suggested that the pancreatic A-cells secreted a hyperglycaemic substance into the portal blood under the stimulation of anterior pituitary growth hormone. (c.f. P.18.



Present experimental work.

Firstly, alloxan diabetic, sclerosed, dog pancreas was prepared (c.f. Section II) and explanted in a biphasic medium in Carrel flasks. Attempts were then made to demonstrate the 'in vitro' secretion by the diabetic, sclerosed pancreas, containing only A-cells and connective tissue, of a hyperglycaemic substance as shown by assay on rabbits. This system was intended to be analogous to a true endocrine secretion. Histological evidence of A-cell rests in the explants and in the sclerosed diabetic pancreas was offered. (c.f. Section III and Appendix 1).

Secondly, an attempt was made to repeat the work of Bornstein, Reid and Young (1951). It was meant to add to it by seeing if growth hormone would still excite secretion of a hyperglycaemic factor after pancreatectomy. (c.f. Section IV).

Results.

The results of the preparation of the dogs are discussed and can be seen in Section II. The results of the tissue culture experiments are recorded and fully discussed in Section III. Briefly, these results were as noted on P.97, i.e.

(1) 2 groups of experiments have been described in which alloxan diabetic, sclerosed dog pancreas was explanted into a biphasic medium in Carrel flasks.

(2) Intravenous injections of supernates and serum-free extracts of supernates from the flasks produce a significant, sustained hyperglycaemia in rabbits.

(3) These results are adduced as further evidence suggesting that the pancreatic A-cells secrete a hyperglycaemic substance, which may be a second pancreatic hormone.

The attempts to prepare A.D.H.A. rats met with failure and consequently the work of Bornstein, Reid and Young (1951) could not be repeated. A.D.H.A. rats have not been successfully prepared by other workers so far as is known, e.g. Best (1953) reported failure to prepare them by the method of Bornstein (1950).

The results of these attempts are fully discussed in Section IV (c.f. P.115).

#### Main Conclusions.

(1) Further evidence has been adduced suggesting that the pancreatic A-cells secrete a hyperglycaemic substance which may be a second pancreatic hormone.

(2) The work of Bornstein, Reid and Young (1951) cannot be repeated owing to inability to prepare the necessary A.D.H.A. rat.

SECTION VI.

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SECTION VII.

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APPENDIX i. (c.f. Section III).

Records of tissue culture experiments (abridged).

14.11.52.

Experiment 1 - 22 explants set up and a further 2 flasks with medium, but no explant (23, 24).

**Tissue:** alloxan diabetic, sclerosed dog pancreas.

Method: Carrel flasks (D.3.5).

Medium: Cockerel plasma 2 parts) 1 c.c.  
Tyrode solution 1 part ) (25 drops from a  
fine Pasteur  
pipette).

Embryo extract (10 day embryo) diluted  
1:4 with Tyrode - 1 drop.

The explants were cut with Borrowdale needles.

Many contained fat.

Flasks placed in copper racks, and incubated at 37°C. in an ordinary bacteriological incubator.

15.11.52. - 7 cultures photographed (c.f. figs. 7 - 13).

Flask 1. Explant healthy. Much fat present. No growth. Clot unremarkable.

2. Explant darkish. No growth. Clot unremarkable.

3. Explant darkish. No growth. Clot unremarkable.

4. Explant healthy. No growth. Clot healthy.
5. Explant healthy. Solid tissue seen in centre of explant. No growth. Clot healthy.
6. Explant healthy. Slight outgrowth (?). Small area of liquefaction of clot at one point.
7. Explant dark. No outgrowth. Clot healthy. Much fat seen.
8. Explant healthy. Solid tissue in centre of explant. No outgrowth. Clot healthy.
9. Explant healthy and composed of solid tissue. Clot healthy.
10. Explant healthy. No outgrowth. Clot healthy. Much fat.
11. Explant darkish. No outgrowth. Clot healthy. Much fat.
12. Explant healthy. No outgrowth. Clot healthy.
13. Explant at side of flask. Observation not possible.
14. Explant healthy. ? slight outgrowth. Clot healthy.
15. Explant dark. No growth. Clot healthy.

16. Explant healthy. No growth. Clot healthy.
17. Explant healthy. Slight outgrowth. Clot unremarkable. Solid tissue is present.
18. Explant healthy. No growth. Clot healthy. A band of (?) connective tissue seen.
19. Explant healthy. No growth. Clot healthy. A band of (?) connective tissue seen.
20. Explant healthy. No growth. Clot healthy. Connective tissue and fat present.
21. Explant healthy. ? slight outgrowth. Clot healthy. Connective tissue and fat present.
22. Explant darkish. No outgrowth. Clot healthy. Fat and connective tissue present.
- 23 Reagent controls - No explant. Media  
&  
24 unremarkable.

16.11.52.

1. Explant healthy. No growth. Slight liquefaction of clot.
2. Explant healthy. No growth. Clot unremarkable.

iv.

3. Fatty part of explant is dark, but a piece of healthy, non-fatty tissue is seen. Clot beginning to liquefy.
4. Some migration of cells noticed at edge of explant, and clot liquefying, but not near explant. Otherwise no change.
5. Explant healthy. No growth. Clot unchanged.
6. Explant dark. Slight segmental outgrowth. Slight area of clot liquefaction near it.
7. Explant dark. No outgrowth. Clot healthy.
8. Non-fatty tissue healthy and liquefaction of clot around it.
9. Explant healthy. No growth. Clot healthy.
10. Explant darkening. No growth. Clot healthy.
11. Explant darkening. No growth. Clot healthy.
12. Explant healthy. No growth. Liquefaction of clot beginning.
13. —
14. Explant healthy. Slight outgrowth. No liquefaction of clot.
15. Explant very dark. No growth. Clot healthy.
16. Explant healthy. No growth. Clot unchanged.



17. Explant healthy. No outgrowth. Clot unchanged:  
ged.
18. Explant healthy. No growth. Clot unchanged.
19. Explant healthy. No growth. Clot shows  
slight liquefaction.
20. Explant healthy. No growth. Liquefaction  
of clot beginning.
21. Explant healthy. No growth. Clot unchanged.
22. Explant darkish. Tissue other than fat  
present and healthy. Clot liquefaction  
beginning.
- 23  
& No change.  
24

All clots patched with 0.5 c.c. 2:1 cockerel  
plasma and Tyrode solution and 1 drop 1:4 embryo  
extract and Tyrode solution.

17.11.52.

1. Explant healthy. No growth. No liquefaction  
of clot.
2. Explant darkening. No growth. No liquefac-  
tion of clot.
3. Explant dark except non-fatty tissue.  
Slight outgrowth. Clot repaired.

4. Explant healthy. No growth. No liquefaction of clot.
5. Explant healthy. No growth. Much non-fatty tissue. Clot healthy.
6. Explant darkening. No growth. Liquefaction of clot repaired.
7. Most of fatty tissue black. Edge of non-fatty tissue alive. No growth. Clot healthy.
8. Explant healthy. No growth. Clot healthy.
9. Explant healthy. No growth. Clot in good condition.
10. Explant darkish. No growth. Clot healthy.
11. Explant dark. No growth. Clot healthy.
12. Explant healthy. No growth. Liquefaction of clot repaired.
14. Explant dark. Definite slight outgrowth. Clot healthy.
13. ---
15. Explant dead.
16. Explant darkish. No growth. Clot no change.
17. Explant moderately healthy. No growth or liquefaction.

18. Explant very dark. No growth. No liquefaction of clot.
19. Explant healthy. No growth. No liquefaction of clot.
20. Explant healthy. No growth. No liquefaction of clot.
21. Explant darkish. No growth. No liquefaction of clot.
22. Non-fatty parts of explant are healthy.
- 23  
& No change.
- 24

Supernatant fluid (human serum 0.5 c.c.  
(Tyrode 0.1 c.c.  
added to every flask.

From now onwards for reasons of brevity the appearances are recorded only of those explants whose supernatant fluids were assayed, and of the reagent control flasks.

18.11.52.

- Flask 4. Explant healthy. Clot not liquefied.
5. Explant healthy. Clot unremarkable.
8. Explant healthy. Clot healthy.
9. Explant healthy. Clot in good condition.
17. Fatty part of explant darkening. Clot healthy.

19. Explant healthy. Clot healthy.

23

& No change.

24

The supernatant fluid from flasks 4, 5 and 9 was removed, pooled and injected intravenously into Rabbit 1, and blood sugars estimated over an hour. (c.f. Graph 4). The fluid was at once replaced by a similar amount of serum - Tyrode (0.5 c.c. human serum and 0.1 c.c. Tyrode solution). This was done throughout whenever a supernatant fluid was removed.

19.11.52.

Flask 4. No change.

5. No change.

8. No change.

9. No change.

17. The non-fatty part is very healthy. Clot is unremarkable.

19. No change.

The supernatant fluid was again removed from flasks 4, 5 and 9, pooled, and injected into Rabbit 1. Blood sugars were estimated over almost 3 hours. (c.f. Graph 5). In every rabbit injected, the subsequent overnight urine was collected and tested for sugar (Benedict) and acetone (Rothera), but none was ever found.

20.11.52.

Flask 4. Explant alive. Clot unchanged.

5. A few necrotic areas are seen in the explant, but most of it is alive. Clot unchanged.

8. Explant quite healthy. No change in clot.

9. Explant a little dark in the centre, but is still light-coloured at the edges. No growth is seen. Clot is firm.

17. Solid part of the explant is very healthy. Trace of fibroblastic outgrowth at edge.

19. Explant is healthy. Slight growth is seen. Clot unchanged.

23  
& No change in clot or supernatant fluid.  
24

The supernatant fluid from flasks 23 and 24 (reagent controls) was removed, pooled and given intravenously to Rabbit 1. The resultant blood sugar curve is seen on graph 6. As usual the fluid was at once replaced in the flasks by the same quantity of a similar serum - Tyrode mixture.

22.11.52.

The supernatant fluid from flasks 8, 17 and 19 was removed, pooled and 1.2 c.c. given intravenously to Rabbit 2. (c.f. Graph 7).

23.11.52.

Flask 4. Explant alive. Clot shows slight liquefaction at edge of explant.

5. Explant appears moderately healthy. Clot has become semi-opaque.

8. Explant healthy. Clot unremarkable.

9. Explant healthy. Clot unremarkable.

17. Explant healthy. A trace of fibrous outgrowth is seen at the edges. Clot healthy.

19. Explant is darkening a little at the centre and the clot is becoming opaque.

23 The clots are becoming opaque, but show  
&  
24 no other abnormality.

The supernatant fluid was taken from flasks 4, 5 and 9, and 1.2 c.c. given intravenously to Rabbit 3. The resultant blood sugar curve is seen on Graph 8. It was not possible to complete this curve as venesection was difficult on this rabbit.

25.11.52.

Pooled supernate from flasks 8, 17 and 19 was given intravenously to Rabbit 2 (c.f. Graph 9) and pooled supernate from flasks 4, 5 and 9 given to Rabbit 4. (c.f. Graph 10). Cultures 4, 5, 9, 8, 17 and 19 were photographed. (c.f. figs. 14 - 19).

It was found the clot had loosened around



cultures 5 and 19. The explants were removed to a hollow ground slide and washed in Tyrode solution. The old clot was trimmed off with Borrowdale needles and the explants subcultured into new D.3.5 flasks.

27.11.52.

0.11 mgm. of crystalline growth hormone (Wilhelmi) in 0.3 c.c. of Tyrode solution was added to each of flasks 4, 9, 8, 17 and 19. The flasks were incubated with the growth hormone for 10 minutes at 37°C. and the supernates were then removed from 4 and 9, pooled and injected into Rabbit 4, (c.f. Graph 12) and pooled from flasks 8, 17 and 19, and given to Rabbit 5. (c.f. Graph 11).

Appearance of cultures.

Flask 4. Explant healthy at the edges. Clot slightly liquefied.

- 5. Explant itself is darkening, and a FUNGUS is seen growing all over the clot.
- 8. Explant darkening. Clot liquefying.
- 9. Explant darkening, and has broken free from the liquefying clot.
- 17. Explant darkening. Spiky growth seen.  
? fungus.
- 19. Explant healthy at edges, but fungus is present.

30.11.52.

All explants fixed in 10% formol saline and sent for histological section.

The reagent control flasks were retained in the incubator.

1.12.52.

Flask 23 - clot liquefied. A fresh reagent control was set up with 25 drops 2:1 cockerel plasma and Tyrode, and labelled flask 25.

2.12.52.

0.6 c.c. 5 parts human serum and 1 part Tyrode solution supernatant fluid added to flask 25.

4.12.52.

Rabbit 1 given 1 c.c. of supernate from flasks 24 and 25 (reagent control). (c.f. Graph 13).  
Rabbit 4 given 1 c.c. of Tyrode solution. (c.f. Graph 14).

8.12.52.

The clots in both flasks 24 and 25 were now liquefied and two further reagent controls were set up - flasks 26 and 27.

9.12.52.

Supernates added to flasks 26 and 27.

16.12.52.

Pooled supernate from flasks 26 and 27 given intravenously to Rabbit 4. (c.f. Graph 15). 1 c.c. of Tyrode solution given intravenously to Rabbit 1. (c.f. Graph 16).

Tissue Culture Micro.      Haematoxylin and Eosin.

Flask 1. Small fragments of necrotic fat and connective tissue only are seen.

3. Fatty fragment with centre destroyed.  
Periphery, though necrotic and showing no nuclei, still maintains its architecture.
4. Connective tissue debris and serous fluid.
5. Necrotic connective tissue and acidophile fluid.
9. Fragments of necrotic connective tissue and fat.
6. Fragments of dead connective tissue and serous fluids.
7. Fragments of collagen.
8. Mycelia and spores seen and some fragments of connective tissue.
10. Fragments of necrotic connective tissue and fat.
11. Fat becoming necrotic, but still showing nuclei.

12. Connective tissue and necrotic fat.
13. Connective tissue and necrotic fat.
14. Connective tissue and necrotic fat.
15. Connective tissue and fragmented fat.
16. Connective tissue and fragmented fat, and also an island of cellular debris.
17. Acidophile debris only.
18. Degenerate fat and connective tissue is seen.
19. Degenerate fat and some connective tissue is seen.
20. Degenerate fat is seen and a few degenerate cellular remnants.
21. Degenerate fat and connective tissue, with a dead blood vessel are seen. Some acidophile cell remnants are noted. Remnants of islet cells ?.
22. Acidophile debris only.

It is thus seen that the tissue explants consisted of connective tissue, which may or may not at an earlier stage have contained functional islets, and fat. The cultures were maintained too long and necrosis of the explants was widespread.

Pancreatic tissue could not be identified in them.

20.10.53.

Experiment 2. - 24 explants of sclerosed pancreas set up. (P.1 - 24). 12 explants of chick heart (H.1 - 12) set up. 12 flasks with plasma clot and supernatant fluid, but NO explant (Reagent control (R.C.) 1 - 12).

(1) Tissue: alloxan diabetic, sclerosed dog pancreas.

Method: Carrel flasks (D.3.5).

Medium: Cockerel plasma 2 parts) 1 c.c. (25 drops  
Tyrode solution 1 part ) from a fine  
Embryo extract (10 day embryo) diluted  
1:4 with Tyrode - 1 drop.

The explants were cut with 2 new single-edged razor blades. The amount of fat present was minimal.

(ii) Tissue: embryonic chick heart (10 day).

Method: )  
Medium: ) as above (i)

(111) 12 flasks with media as above, but no explants.

The flasks were incubated at 37°C.

21.10.53.

A number of explants were photographed (figs. 20 - 25).

H.1 - 12: All the heart tissue is beating and healthy. All explants show slight out-growth. Clots are healthy.

R.C.1 - 12: Clots are firm and healthy.

P.1: Explant healthy in suspension. No growth.

Clot liquefied.

2: Explant healthy and still attached to some clot. Most of clot liquefied.

3: Explant healthy and still attached to some clot. Most of clot liquefied.

4: Explant healthy and freely mobile. Clot liquefied.

5: Explant healthy and freely mobile. Clot liquefied. A small piece of fat is attached to explant.

6: Explant healthy and firmly fixed. Clot largely liquefied.

7: Explant mobile and healthy. Clot completely liquefied.

8: Explant mobile and healthy. Clot completely liquefied.

9: Explant mobile and healthy. Clot completely liquefied.

10: Explant mobile and healthy. Clot partially liquefied.



- 11: Explant healthy. Slight liquefaction of clot, but it has retained discoid form, although it is loose.
- 12: Explant not clearly seen. Opacity around it. Clot very slightly liquefied.
- 13: Explant healthy and mobile. Clot liquefied.
- 14: Explant healthy and mobile. Clot completely liquefied.
- 15: Explant healthy and mobile. Clot liquefied.
- 16: Explant healthy and mobile. Clot liquefied.
- 17: Explant healthy, but small. Is mobile. Clot liquefied.
- 18: Explant healthy. Clot liquefied.
- 19: Explant healthy and a cuff of growth is seen around it. Clot liquefied.
- 20: Explant healthy and a cuff of growth is seen around it. Clot liquefied.
- 21: Explant healthy, but small. Clot liquefied.
- 22: Explant healthy. Clot liquefied.
- 23: Explant healthy. Clot liquefied.
- 24: Explant healthy. Clot liquefied.

The pancreatic explants only in the majority of cases have liquefied the clot. Fluid (cockerel serum) removed from all flasks, pooled and stored at  $-25^{\circ}\text{C}$ . All liquefied clots were now patched. This was done using 25 drops 2:1 cockerel plasma and Tyrode, with 1 drop 1:4 embryo extract. Explants P.13 - 18 were sacrificed for histology

and fixed in 10% formol saline.

22.10.53.

- P.1. Explant healthy. No liquefaction of the clot.
2. Explant healthy. Slight liquefaction causing whole clot to move.
  3. Explant healthy. Clot firm.
  4. Explant healthy. Slight liquefaction of the clot.
  5. Explant healthy. Slight liquefaction of the clot.
  6. Explant healthy. Slight liquefaction of the clot.
  7. Explant healthy. Clot firm.
  8. Explant healthy. Slight liquefaction of clot.
  9. Explant healthy. Slight liquefaction of clot.
  10. Explant healthy. Slight liquefaction of clot.
  11. Explant healthy. Slight liquefaction of clot.
  12. Much cloudiness is seen around the explant and migration of cells. Clot not liquefied.
- P.19, 20, 21, 22, 23 and 24 show slight liquefaction of the clot, but in general liquefaction is not so pronounced as after first day of incubation and explants are held in a slightly liquefied, moving disc of clot.

To all cultures 15 drops 5:1 human serum and Tyrode solution were added. Sacrifice P.19 - 24 for histology.

H.1 - 4 and 6 - 12. All beating healthily and showing outgrowth.

H.5. is slightly darker, but showing outgrowth.

R.C. 1 - 12. Nothing to note.

24.10.53.

P.1 - 12. Are all darkening.

P.12 shows turbidity of supernate and is infected.

The supernate should not be pooled with the supernates of P.1 - 11.

H.1 - 4. Healthy, growing and beating.

H.6. Healthy, growing, but not beating.

H.5. Explant is darkening. Slight growth is present.

H.7 - 12. Healthy, growing and beating.

R.C.1 - 12. Nil to note.

All pancreatic supernates 1 - 11 were pooled.

All heart supernates 1 - 12 were pooled.

All reagent control supernates 1 - 12 were pooled.

All explants were fixed in 10% formol saline and sent for section.

The 3 lots of pooled supernate together with the pooled supernate from 21.10.53 were stored at -25°C. until they could be sent for extraction of the hyperglycaemic factor. There were thus 4 lots of pooled supernates.

(1) Second day pooled pancreatic supernate;

(2) Fifth day pooled pancreatic supernate.

- (3) Fifth day pooled chick heart supernate;
  - (4) Fifth day pooled reagent control supernate.
- (For method of extraction c.f. Section III).

The extracts were received back from Dr. Gilchrist and given intravenously as seen on Graphs 22 - 29, and as described in Section III, Experiment 2. Urine was examined for sugar 20 hours after injection and none was found in any instance.

Sclerosed, diabetic pancreatic explants -

Microscopic appearance of sections of explants.

Haematoxylin and Eosin.

P.1. This explant consists of vascular fibrous granulation tissue chiefly, with some haemosiderin deposition. There is, in one area, a configuration of reticular material which might have surrounded acini, but it is not now possible to say if this is so. In a tiny cleft in the granulation tissue a small basophil concretion is seen. The explant is surrounded by a rim of acidophile serous fluid (fig. 26).

Gomori: One or two little nests of pink staining cells are seen which may be A-cell islets.

P.2. Haematoxylin and Eosin. Explant consists chiefly of fibrous granulation tissue penetrated by many vessels and a few nerves. It is infiltrated by numbers of chronic inflammatory cells, chiefly histiocytes, many of which

contain haemosiderin. This latter substance is also lying in profusion in the interstitial tissues. No acinar remnants or islet tissue is seen with this stain.

Gomori. Islet tissue and acinar remnants not identified certainly, but one or two groups of cells suggestive of the former are seen.

P.3. Haematoxylin and Eosin. Explant consists of oedematous, fibrous granulation tissue only.

Gomori. Appearance as in Haematoxylin and Eosin. There is no evidence of pancreatic remnants or of islet tissue.

P.4. Haematoxylin and Eosin. Central necrosis of the explant is seen. The tissue remaining alive more peripherally, shows a few pancreatic ductules and a few atrophic acini surrounded by fibrous tissue. The viable rim is heavily infiltrated by round cells and many blood vessels are seen as well as autonomic nerve bundles (fig. 27).

Gomori. One group of acidophil cells is seen highly suggestive of A-cell islet tissue.

P.5. Haematoxylin and Eosin. Section consists of oedematous fibrous granulation tissue investing the atrophic remnants of pancreatic acini. The edge of the explant shows a rim of acidophile serum and plasma clot.

Gomori. Islet tissue is seen at one or two places. A few A-cells surrounded by collagenous material are noted. Red granules are seen in the A-cells (fig. 28).

P.6. Haematoxylin and Eosin. Explant is largely fibrous granulation tissue with a small necrotic area at the centre. There are, however, some atrophic remnants of acini seen, although there is no islet tissue to be identified by this stain.

Gomori. Acinar remnants are seen, but no islet tissue is identified on this section.

P.7. Haematoxylin and Eosin. This is a small piece of tissue consisting of fibrous tissue and round cells and some acinar remnants. Some haemosiderin is lying free in this granulation tissue.

Gomori. The serous fluid on the surface of the explant has stained red, but no cells have done so.

P.8. Haematoxylin and Eosin. Consists of fibrous granulation tissue infiltrated by inflammatory cells. Some atrophic pancreatic ductules are distinguished surrounded by much fibrosis. Haemosiderin is seen deposited throughout the explant.

Gomori. No islets identified.



P.9. Haematoxylin and Eosin. Section shows fibrous granulation tissue infiltrated by inflammatory cells. Some acinar remnants can be distinguished.

Gomori. Islets are not seen.

P.10. Haematoxylin and Eosin. Fibrous granulation tissue only. No pancreatic remnants identified.

Gomori. No islets identified and no acinar remnants seen.

P.11. Haematoxylin and Eosin. Explant consists of fibrous granulation tissue and atrophic acini. Some adherent clot is attached to the edges of the explants (fig. 29).

Gomori. No islets seen.

P.12. Haematoxylin and Eosin. Consists of fibrous granulation tissue only. The culture appears to be infected by sporing anaerobic bacilli and is partially necrotic.

Gomori. Pancreatic remnants can be seen, but islets are not identified.

P.13. Haematoxylin and Eosin. Explant consists of oedematous fibrous granulation tissue. There is no pancreatic tissue seen.

Gomori. No pancreatic tissue seen.

P.14. Haematoxylin and Eosin. Explant consists of oedematous fibrous granulation tissue and autonomic nerves surrounding atrophic

pancreatic remnants.

Gomori. Atrophic acini are seen, but no islets are found.

P.15. Haematoxylin and Eosin. Explant consists of fibrous granulation tissue: pancreatic tissue is not seen.

Gomori. No pancreatic tissue seen.

P.16. Haematoxylin and Eosin. Explant consists of fibrous granulation tissue containing a number of polymorphs. Pancreatic tissue not seen.

Gomori. No pancreatic tissue seen.

P.17. Haematoxylin and Eosin. Consists of fibrous granulation tissue only. No obvious pancreatic tissue remains.

Gomori. No islets are found.

P.18. Haematoxylin and Eosin. Fibrous granulation tissue only is seen and no pancreatic remnants distinguished.

Gomori. No islets, but some acinar remains identified.

P.19. Haematoxylin and Eosin. Fibrous granulation tissue only is noted. There is no pancreatic tissue seen.

Gomori. No pancreatic tissue found.

P.20. Haematoxylin and Eosin. Fibrous granulation tissue only is seen.

Gomori. No pancreatic tissue made out.

P.21. Haematoxylin and Eosin. Shows fibrous granulation tissue surrounding acinar remnants. On the surface of the explant is some fatty tissue.

Gomori. No islets seen.

P.22. Haematoxylin and Eosin. Fibrous granulation tissue surrounding acinar remnants. Some fatty tissue included.

Gomori. No islets seen.

P.23. Haematoxylin and Eosin. Acinar remnants surrounded by fibrous granulation tissue. Haemosiderin present.

Gomori. One possible islet seen.

P.24. Haematoxylin and Eosin. Acinar remnants and fibrous granulation tissue also. Necrosis is beginning at the centre with break up of cell nuclei.

Gomori. No islets seen.

Chick heart explants.

- H.1. Haematoxylin and Eosin. Most of explant healthy with a little central necrosis.
- H.2. Haematoxylin and Eosin. Incipient necrosis in one area, but most is healthy.
- H.3. Haematoxylin and Eosin. Necrosis at centre of the explant, and at periphery the heart cells are forming a proliferating, flat, syncytial sheet.
- H.4. Haematoxylin and Eosin. Necrosis at centre of the explant, and at periphery the heart cells are forming a proliferating, flat syncytial sheet, and in addition vacuolation of peripheral cells is seen. Some acidophile material is noted laid down between them.
- H.5. Haematoxylin and Eosin. Only acidophil fluid is seen, but no tissue is present.
- H.6. Haematoxylin and Eosin. Consists of a ramifying network of fibroblasts. Some areas of necrosis are seen and some sheets of proliferating heart muscle cells and fibroblasts.
- H.7. Haematoxylin and Eosin. Some central necrosis is seen, but the peripheral part of the explant is healthy.

- H.8. Haematoxylin and Eosin. Explant healthy. Much fibroblastic proliferation is seen.
- H.9. Haematoxylin and Eosin. Explant healthy. Much fibroblastic proliferation is seen.
- H.10. Haematoxylin and Eosin. One area of necrosis is seen. Peripherally there is much proliferation of fibroblasts and heart cells.
- H.11. Haematoxylin and Eosin. Explant is healthy throughout. Some outgrowth is seen.
- H.12. Haematoxylin and Eosin. Explant is healthy throughout. Some outgrowth is seen.

APPENDIX 11 a. (c.f. Section II).CASE HISTORIES AND AUTOPSY FINDINGS ON DOGS.

Dog 1.           Variety - Collie.  
                  Weight - 12.5 Kg.

20.6.52.

10.10 hours. Fasting blood sugar = 74 mgm.%.

10.15 hours. 50 mgm./Kg. of body weight alloxan  
was given intravenously in 5% solution of distilled  
water.

14.30 hours. Blood sugar = 114 mgm.%.

21.6.52.

Animal appears healthy clinically.

09.30 hours. Fasting blood sugar = 60 mgm.%.

23.6.52.

09.50 hours. Fasting blood sugar = 178 mgm.%.

Animal shows abnormal thirst, but is otherwise  
quite healthy.

14.15 hours. Operation to ligate right limb of  
pancreas. (c.f. Section II).

24.6.52.

Animal in poor spirits. Drinks a good deal and  
vomits sometimes.

27.6.52.

Looks very much better.



28.6.52.

Quite fit and playful. Some excoriation of the wound. Penicillin powder applied. Drinking a little less now.

5.7.52.

Quite recovered from operation. Wound healed.

15.7.52.

Still drinking excessively - weight falling = 10.8 Kg.

4.8.52.

09.30 hours. Fasting blood sugar = 178 mgm.%. Drinks excessively.

5.8.52.

Urinary sugar (Benedict) = orange (++).

13.8.52.

Glucose tolerance test performed (c.f. Graph 1).

8.10.52.

Dog now extremely thin.

10.11.52.

10.00 hours. Fasting blood sugar = 99 mgm.%.

Dog very thin and vomiting. Appears semi-comatose. Given 50,000 units penicillin at once and then twice daily.

Given 5 units globin insulin at once and then to have 5 units each day before its meal. Glucose and water to be added to meal.

11.11.52.

Dog appears better.

10.10 hours. Fasting blood sugar = 159 mgm.%.

12.11.52.

Dog appears quite fit again. Insulin stopped.

14.11.52.

Operation. Animal given 0.5 G. of sodium thiopentone quickly intravenously, from which he died. Abdomen opened and the ligated part of the pancreas identified from the ligatures still 'in situ'. The ligated limb consisted of fatty fibrous tissue and the main vessels and remnants of the duct were seen. The alloxan diabetic, sclerosed pancreatic tissue, removed aseptically, was put at once into Tyrode solution in a test-tube and removed to the tissue culture room, where it was put up as described in Section III, Experiment 1. Unligated diabetic pancreas and sclerosed pancreas were fixed immediately in 10% formol saline. 5 hours later a complete post-mortem was performed.

Dog 1.      Post Mortem - 14.11.52.

Weight: 9.5 Kg.      P.M. 5 hours after death.

(Pancreas fixed 5 minutes after death)

GENERAL APPEARANCE:

The dog was emaciated and had lost a great deal of weight. No other external pathological signs.

SEROUS SACS:

Nil abnormal seen.

ENDOCRINE SYSTEM:

Thyroid was healthy.

Suprarenals were healthy.

The right limb of the pancreas had been ligated and as far as could be seen consisted entirely of fatty, fibrous tissue lying to the left (when reflected) of the second part of the duodenum and attached to it by a short mesentery of 1.5 cms. breadth. The main pancreatic vein and the remnants of the duct were identified on the back of this fatty fibrous mass. The point of ligature and division was identified by the black, silk threads still seen 'in situ'. The remainder of the pancreas appeared to have hypertrophied and weighed in all 28 gms. There was no abnormality seen naked eye in this.

ALIMENTARY SYSTEM:

Oesophagus, stomach, duodenum and intestines were healthy.

The liver was of normal size, but extremely soft and on section appeared a greyish-pink colour and was almost of a liquid consistence when cut.

Gall bladder and bile ducts: N.A.D.

RESPIRATORY SYSTEM:CARDIOVASCULAR SYSTEM:

) Nil abnormal seen.

UROGENITAL SYSTEM:

Nothing abnormal to be seen naked eye in kidneys, pelvis, ureters or bladder.

Urine was removed from the bladder at post mortem and was found on testing with Benedict's solution to reduce it to a brick-red colour (+++). The Rothera's test for acetone was strongly positive.

Microscopic Report

Heart: No abnormality seen.

Lung: Groups of histiocytes are noted in the interstitial tissue, here and there. Slight congestion is the only other feature.

Spleen: A great deal of light brown pigment occurs in the sinuses and pulp. Otherwise there is no abnormality.

Prussian Blue Reaction - The pigment stains the blue colour of iron. This is frequently seen in the dog spleen.

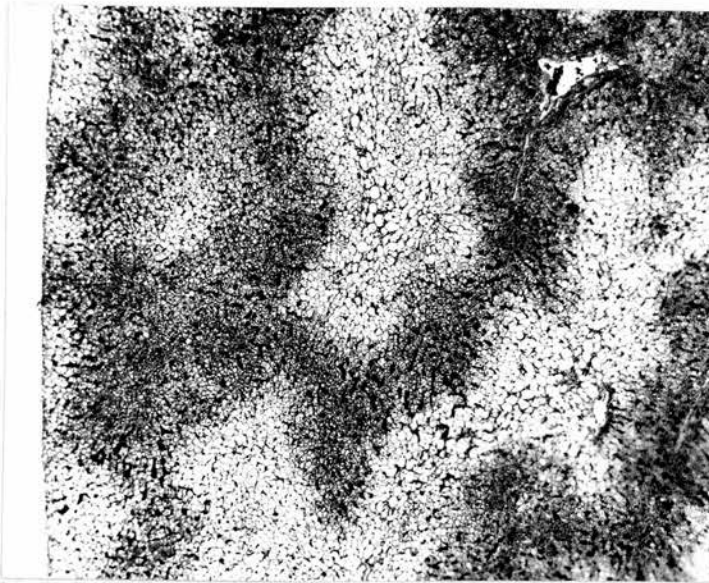


Fig. 34.

Dog 1. Liver. Haematoxylin and Eosin.  
x 40. A marked degree of centri-lobular  
fatty degeneration - alloxan diabetes and  
sclerosis of part of pancreas.

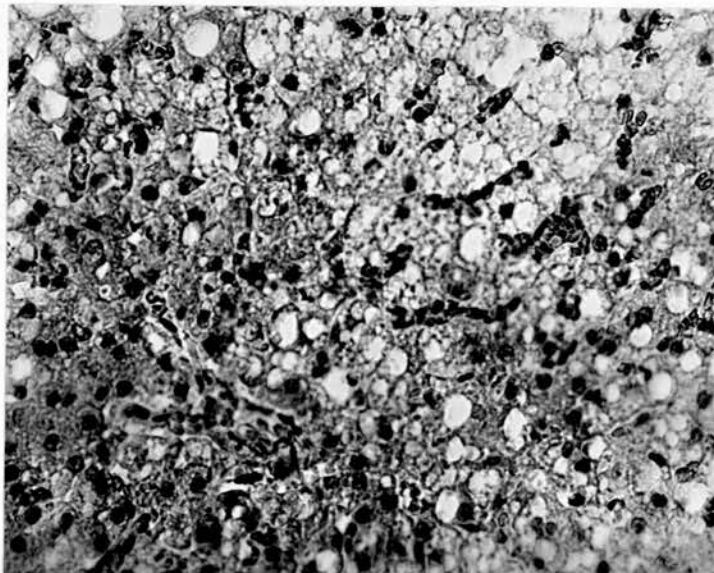


Fig. 35.

Dog 1. Liver. Haematoxylin and Eosin.  
x 350. Higher power of liver cells  
showing fatty vacuolation.

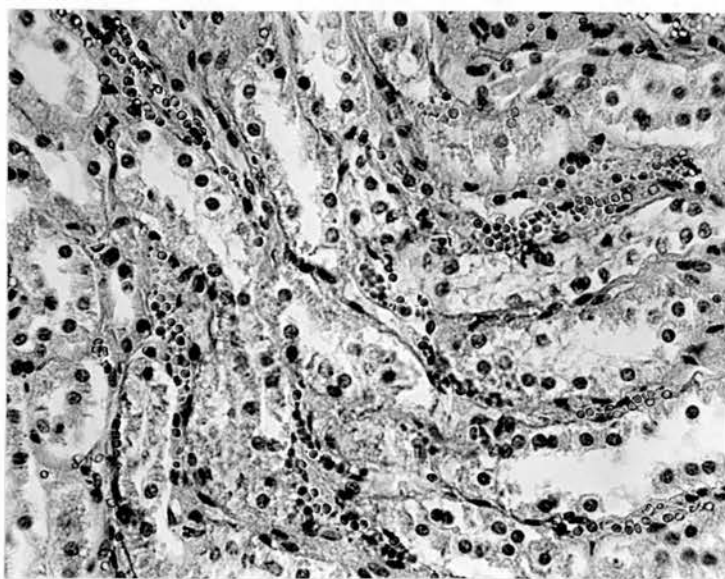


Fig. 36.

Dog 1. Kidney. Haematoxylin and Eosin.  
x 300. Fatty vacuolation of distal  
tubules - alloxan diabetes.



Fig. 37.

Dog 1. Pancreas (unligated). Haematoxylin  
and Eosin. x 200. The islets are reduced  
in size and have a crescentic or linear  
shape - alloxan diabetes.



Liver: An extreme degree of fatty degeneration occurs. This is most marked in the centres of the lobules, which are almost entirely replaced by fatty droplets. Only occasional liver cells near the portal tracts have escaped damage. (figs. 34 and 35).

Periodic Acid Schiff: - McManus: The vacuoles do not stain as polysaccharide.

Kidney: The only pathological change noted is vacuolation of the cells of Henle's loop and distal convoluted tubules (fig. 36).

Suprarenal: Zona of the cortex are very well demarcated from each other and show no abnormality. The medulla is healthy.

Small intestine (jejunum): No abnormality is seen, although lymphoid follicles appear moderately reactive.

Pancreas: (unligated). Haematoxylin and Eosin. The islets are greatly reduced in size and many have a crescentic or linear shape. Their number does not appear much altered. (fig. 37). Some hydropic change of remaining islet cells is seen. (fig. 38). This may have resulted from the prolonged hyperglycaemia (5 months). No abnormality of acinar tissue, ducts or fibrous framework is noted. Gomori. The islets are seen to consist entirely of cells with basophilic nuclei and pinkish red cytoplasm. B-cells are infrequently seen, but here and there they do occur. Red granules can be made out in the A-cells.

Pancreas: (ligated). Haematoxylin and Eosin. A small amount of pancreas remains. One or two nodules of atrophic, fibrosed acinar tissue can be distinguished in the sections. Islets cannot be identified. Extensive fatty replacement of pancreas has occurred. (figs. 4 and 5).

Gomori. Islets are not distinguished.

### F i n a l   A b s t r a c t .

Alloxan diabetes of 5 months standing.  
Successful ligation of right limb of pancreas with subsequent atrophy.



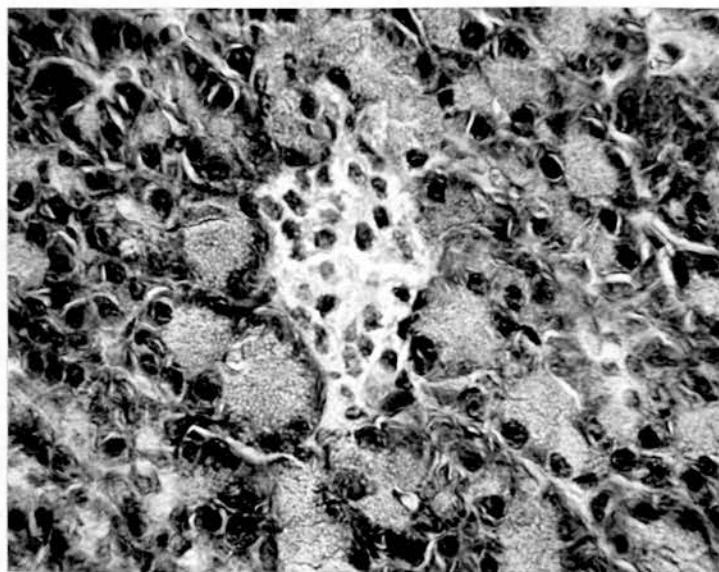


Fig. 38.

Dog 1. Pancreas (unligated). Haematoxylin and Eosin. x 575. Hydropic change in cells of a small islet following 5 months hyperglycaemia - alloxan diabetes.

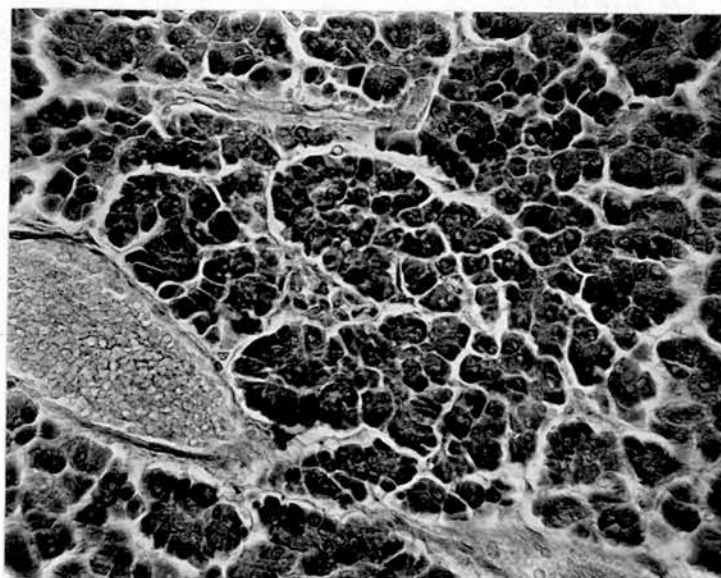


Fig. 39.

Dog 3. Pancreas. Gomori. x 300. Small islet consisting of A-cells - alloxan diabetes.

Dog 3.      Variety - Cairn Terrier (bitch).  
Weight - 8.5 Kg.

13.8.52.

A glucose tolerance test was performed (c.f. Graph 2).

14.8.52.

10.15 hours. Fasting blood sugar = 65 mgm.%.  
Alloxan administered, 60 mgm./Kg. of body weight  
intravenously in 5% solution of distilled water.  
15.35 hours. Fasting blood sugar = 105 mgm.%.

15.8.52.

09.45 hours. Fasting blood sugar = 65 mgm.%.

16.8.52.

Died during night (Saturday). Autopsy not  
performed until Monday morning.

Dog 3.      Post Mortem - 18.8.52.

No external pathological signs seen.

SEROUS SACS:

The mesentery appeared to contain some gelatinous fluid.

ALIMENTARY SYSTEM:

Oesophagus, stomach and intestines were healthy.

The liver showed no abnormality.

The gall bladder was healthy.

RESPIRATORY SYSTEM:

Larynx, trachea and bronchi were healthy.

The left lower lobe appeared congested and consolidated. The remainder of the lungs was healthy.

CARDIOVASCULAR SYSTEM:

No abnormality seen.

RETICULO-ENDOTHELIAL SYSTEM:

The spleen appeared healthy.

No abnormality of lymph nodes seen.

ENDOCRINE SYSTEM:

The suprarenals appeared healthy.

Pancreas was normal naked eye. It weighed 17 g.

UROGENITAL SYSTEM:

Both kidneys were normal in shape and size. On section there was no abnormality.

Uterus and uterine adnexae were healthy.

Microscopic Report

Pancreas: Haematoxylin and Eosin. The parenchymatous cells are engorged with granules and the small ducts contain acidophil secretion. Few islets are seen and these are much reduced in size. Autolysis is present in some areas.

Gomori. A predominance of pink staining cells is seen in the islets with a few having bluish cytoplasm. A few of these cells contain similarly coloured granules, but most do not. (fig. 39).

Lying in the adventitia of the smallest ducts are cells morphologically identical with islet cells, with bluish staining cytoplasm. They do not contain granules.

Lung: Terminal congestion and oedema.

Liver: Terminal congestion and oedema. The liver cells show fine fatty vacuolation throughout with no special distribution.

Periodic Acid Schiff: No polysaccharide is stained.

Heart: No abnormality seen.

Suprarenal: No abnormality seen.

Spleen: Some congestion is present. A fair amount of free haemosiderin occurs.

Kidney: Autolysis of proximal convoluted tubules is seen. Considerable vacuolation of cells in the distal convoluted tubules and in the ascending limb of the loop of Henle is present, with nuclei still preserved.

Periodic Acid Schiff. (McManus). Basement membranes stain, but not the vacuoles in distal convoluted tubules.

### F i n a l     A b s t r a c t .

Death from diabetic-uraemic syndrome, due to alloxan.

Dog 4.     Variety - Brindled fox terrier.

Weight - 9 Kg.

2.10.52.

10.45 hours. Fasting blood sugar = 98 mgm.%.

10.46 hours. Alloxan administered intravenously, 60 mgm./Kg. of body weight in 5% solution of distilled water.

16.00 hours. Blood sugar = 87 mgm.%.

3.10.52.

10.20 hours. Fasting blood sugar = 245 mgm.%.

4.10.52.

10.00 hours. Fasting blood sugar = 183 mgm.%.

9.10.52.

10.00 hours. Fasting blood sugar = 192 mgm.%.

10.10.52.

10.00 hours. Fasting blood sugar = 292 mgm.%.

16.10.52.

Operation - To ligate pancreatic ducts.

The animal was anaesthetised with 0.25 G. of sodium thiopentone and chloroform. The abdomen was opened through a midline upper abdominal incision. The pancreas and duodenum were eviscerated. The common bile duct was located as it passed into duodenum. The pancreatic ducts did not join it. The vessels between head of pancreas and duodenum were ligatured and divided, and blunt dissection was made on both sides of the entrance of common bile duct. A large pancreatic duct was found on the right side of the entrance of common bile duct. A duct, smaller than the first, was found lateral to that. Both ducts were ligated and divided. A search was made for other ducts, but none was found. The duodenum, on returning to the abdomen, appeared cyanosed, and it was considered that ligation of pancreatoco-duodenal vessels had been too extensive and that gangrene might supervene. Abdomen closed in layers after sprinkling streptomycin on operation area.

17.10.52.

10.00 hours. Has drunk 250 c.c. milk, looks very ill.

15.00 hours. Died.

Dog 4.            Post Mortem - 17.10.52.

(P.M. 3 hours after death).

GENERAL APPEARANCE:

The only external pathological sign seen was some 5 - 10 c.c. of bloodstained, turbid fluid in the operation wound.

SEROUS SACS:

Peritoneal Sac: The peritoneum was injected throughout, and the bowel beneath it was reddened.

Pleural Sac and Pericardial Sac: Nil abnormal found.

ALIMENTARY SYSTEM:

Stomach was healthy.

The second part of the duodenum appeared blue, suggesting early gangrene. The remainder of the gut was normal, apart from the injection described.

The liver appeared healthy.

The gall bladder was healthy.

UROGENITAL SYSTEM:

Kidneys, pelvis and ureters: unremarkable.

The bladder was healthy.

RETICULO-ENDOTHELIAL SYSTEM:

The spleen appeared normal. No abnormality of lymph nodes.

CARDIOVASCULAR SYSTEM:

No abnormality of the chambers, valves or heart wall. The vessels were healthy.

RESPIRATORY SYSTEM:

Trachea and bronchi and lungs were normal.



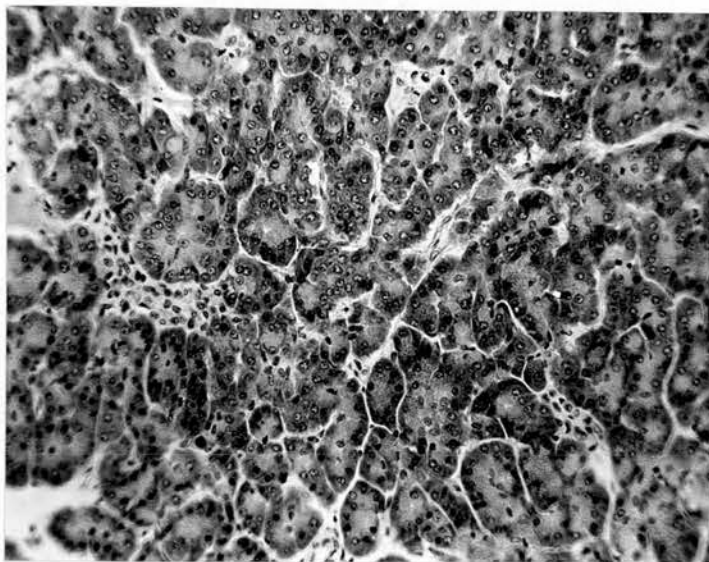


Fig 40.

Dog 4. Pancreas. Haematoxylin and Eosin.  
x 200. Islets are reduced in size and of  
crescentic shape - alloxan diabetes.

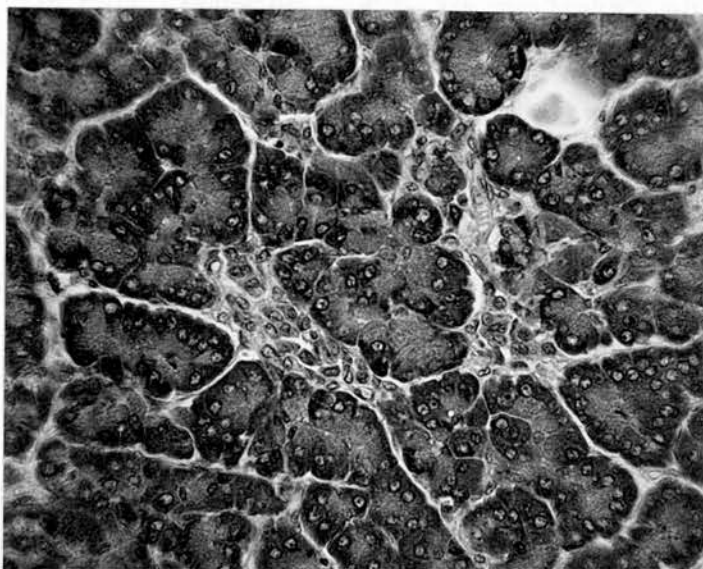


Fig. 41.

Dog 4. Pancreas. Gomori. x 300. Islets  
consisting entirely of A-cells - alloxan  
diabetes.

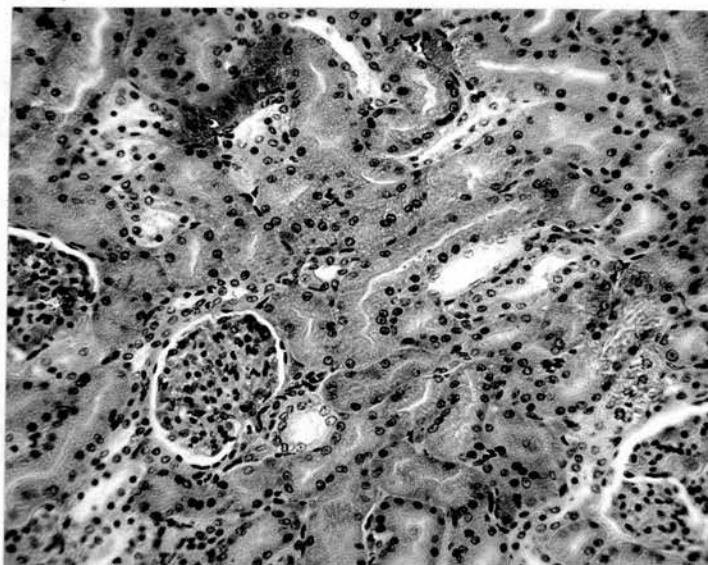


Fig. 42.

Dog 4. Kidney. Haematoxylin and Eosin.  
x 200. Kidney unaffected by alloxan.

Microscopic Report.

Pancreas: The fibrous septa are infiltrated heavily with polymorphs.

The islets are seen to be much reduced in size, and most of them consist entirely of A-cells whose granules are stained red by Gomori stain. In some islets, however, B-cells are seen with blue-black granules. (figs. 40 and 41).

Ligated Channel: Shows a little pancreas and a main pancreatic duct. (Removed to check correct identification of largest pancreatic duct during operation).

Suprarenal: No abnormality.

Spleen: Some autolysis is seen, but no other abnormality.

Lung: Terminal congestion and oedema only is seen.

Liver: There appears to be a centrilobular foaminess leaving a rim of more healthy liver cells around the portal tracts. A great deal of bile is deposited in these central areas. These changes may be associated with the administration of chloroform.

Kidney: No abnormality is seen. (fig. 42).

Final Abstract.

Death occurred from post-operative shock and gangrene of the bowel in an alloxan diabetic animal.

Dog 5. Variety - Brown cocker spaniel (bitch)

Weight - 11 Kg.

23.10.52.

10.15 hours. Fasting blood sugar = 89 mgm.%.

10.20 hours. 60 mgm. alloxan/Kg. of body weight given intravenously in 5% solution of distilled water over 2 minutes.

15.30 hours. Blood sugar = 76 mgm.%.

24.10.52.

10.10. hours. Fasting blood sugar = 103 mgm.%.

25.10.52.

10.00 hours. Fasting blood sugar = 96 mgm.%.

30.10.52.

10.00 hours. Fasting blood sugar = 95 mgm.%.

31.10.52.

Urinary sugar (Benedict) = negative.

5.11.52.

11.15 hours. Fasting blood sugar = 92 mgm.%.

Alloxan, 60 mgm./Kg. of body weight was given intravenously.

6.11.52.

10.15 hours. Fasting blood sugar = 117 mgm.%.

7.11.52.

10.00 hours. Fasting blood sugar = 92 mgm.%.

10.11.52.

Urinary sugar (Benedict) = negative.

It is concluded this animal is not diabetic and should be discarded.

Dog 6.                      Variety - Collie.  
Weight - 12.5 Kg.

6.11.52.

10.30 hours. Fasting blood sugar = 79 mgm.%.  
60 mgm. alloxan/Kg. of body weight in 5% solution  
of distilled water given intravenously in 2  
minutes.

16.15 hours. Blood sugar = 199 mgm.%.

7.11.52.

10.00 hours. Fasting blood sugar = 376 mgm.%.

10.11.52.

10.30 hours. Fasting blood sugar = 675 mgm.%.

11.11.52.

10.00 hours. Fasting blood sugar = 700 mgm.%.

12.11.52.

Dog appeared quiet, but reasonably well at 10.00  
hours, but was found dead at 14.30. Rigor mortis  
generalised.

Dog 6.                      Post Mortem - 12.11.52.

(P.M. 4 hours after death).

EXTERNAL APPEARANCE:

The dog had evidently been vomiting and  
dark-stained faeces had been passed.

SERIOUS SACS: Nil abnormal seen.

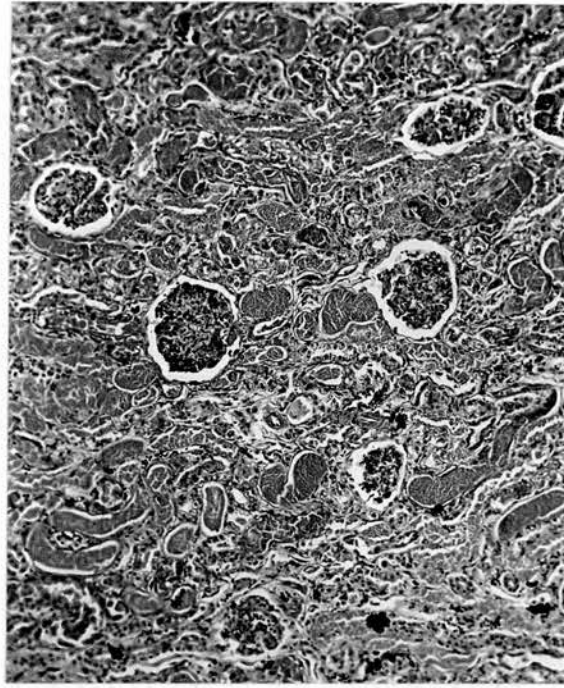


Fig. 43.

Dog 6. Kidney. Haematoxylin and Eosin.  
x 85. Severe damage to convoluted tubules  
caused by alloxan.

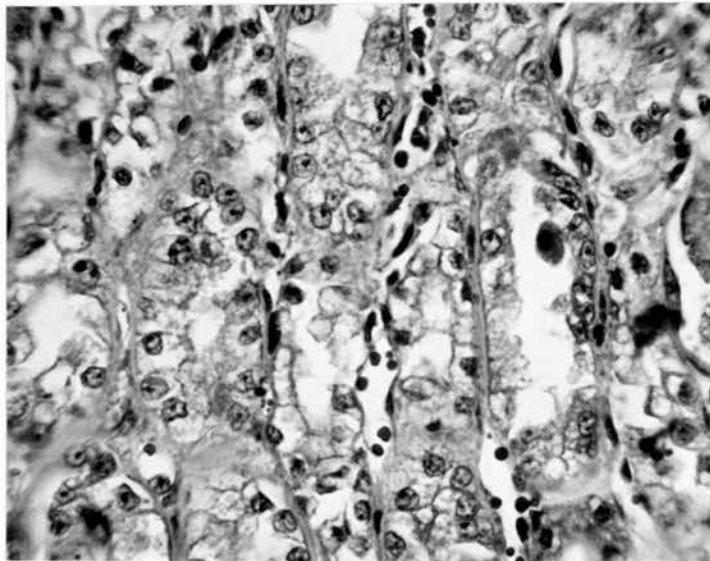


Fig. 44.

Dog 6. Kidney. Haematoxylin and Eosin.  
x 450. Fatty vacuolation of the ascending  
limb of Henle's loop due to alloxan.



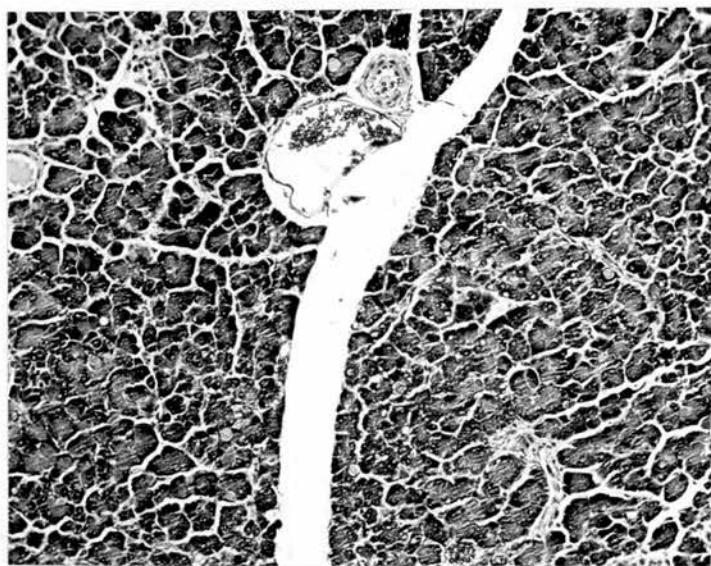


Fig. 45.

Dog 6. Pancreas. Haematoxylin and Eosin. x 110. Islets reduced in size - alloxan diabetes.

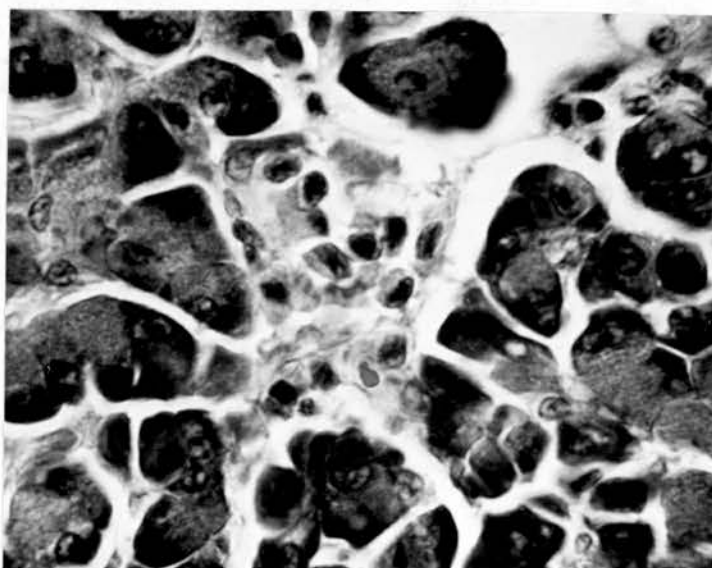


Fig. 46.

Dog 6. Pancreas. Gomori. x 675. Small islet showing central necrosis and a rim of surviving A-cells at the periphery - alloxan diabetes.

ALIMENTARY SYSTEM:

Mouth, tongue, oesophagus, stomach and duodenum appeared healthy.

3 easily reduced intussusceptions were present in the small intestine. They did not appear to be oedematous, and the bowel showed no signs of damage or even congestion. The remainder of the gut was healthy.

The liver appeared exceedingly friable, and on section parts of it were almost fluid.

The gall bladder was dilated as was the common bile duct.

The pancreas was a large one, and weighed 30 G. No external abnormality could be seen in it.

RESPIRATORY SYSTEM:

Larynx, trachea, bronchi and lungs: N.A.D.

CARDIOVASCULAR SYSTEM: N.A.D.

ENDOCRINE SYSTEM:

Thyroids were examined, but no abnormality was seen.

The left suprarenal appeared normal; the right suprarenal seemed a little enlarged.

The pancreas was as described above.

UROGENITAL SYSTEM:

No abnormality seen.

RETICULO-ENDOTHELIAL SYSTEM:

The spleen appeared healthy.

No enlarged lymph nodes were found.

Microscopic Report

Heart: No abnormality seen.

Lung: No abnormality seen.

Liver: Acute congestion is present. Almost all the liver cells are foamy or show definite fatty

vacuolation. Increase of round cells, particularly plasma cells, is noted in some portal tracts.

In some sections the centri-lobular vacuolation and foaminess amount almost to atrophy. It is, however, not so marked as in Dog 1.

Periodic Acid Schiff: (McManus). The vacuoles in the liver cells do not stain as polysaccharide.

Spleen: A small quantity of iron pigment is seen in the splenic substance, but otherwise there is no abnormality.

Kidney: All parts of the tubules are affected, but most markedly the convoluted tubules which show coagulative necrosis and less markedly Henle's loop, which nevertheless displays severe fatty degeneration. These latter tubules contain necrotic epithelial and granular casts.

Periodic Acid Schiff: (McManus). Vacuoles do not stain.

Periodic Acid Schiff: (Feulgen). Vacuoles do not stain.

These changes are very extensive; hardly a nephron has escaped. (figs. 43 and 44).

Suprarenal: No abnormality seen.

Thyroid: No abnormality seen.

2 parathyroids are seen on this section, and are quite normal.

Submaxillary Salivary Gland: No abnormality seen.

#### Pancreas:

Head - Haematoxylin and Eosin. A few islets are seen, reduced in size. The acinar cells are disarticulated, and so are the islets, although the cells themselves are well-preserved. (fig. 45).

Gomori. The islets are seen to be much reduced in size and their architecture disrupted. The cells of which they are composed are filled by red granules, showing they are A-cells. B-cells containing blue granules are not seen. (fig. 46).

Tail - Haematoxylin and Eosin. The islets appear to be reduced in size and number.

Gomori. Such islets as can be found consist almost entirely of A-cells.

#### F i n a l   A b s t r a c t .

Death from diabetic-uraemic syndrome due to alloxan.

Dog 7. Variety - Old English Sheepdog Type (bitch).

Weight - 14 Kg.

7.1.53.

Operation - Complete ligation of pancreatic ducts.

Anaesthesia was induced with 0.25 G. sodium thiopentone and chloroform. This failed to anaesthetize sufficiently. Change to ether also failed and additional sodium thiopentone was required.

The duodenum and head of pancreas were fixed firmly to the posterior abdominal wall, and access was difficult. The common bile duct could not be found after exhaustive search, but 2 pancreatic ducts were located entering duodenum. These were ligated and divided. In addition, the whole of the right limb was ligated and divided. In the dissection, attempting to find the common duct, a good deal of haemorrhage occurred, and the animal was eviscerated for a considerable period. The abdomen was closed in layers with difficulty. Operation time - 3 hours. Dog had penicillin 300,000 units and streptomycin 0.25 G. twice daily.

The dog appeared in fairly good condition at the end in spite of the heavy trauma.

8.1.53. Died overnight.

Dog 7.

Post-Mortem - 8.1.53.

(P.M. 4 - 18 hours after death).

GENERAL APPEARANCE: Nil to note.

SEROUS SACS:

The peritoneal sac contained approximately 40 c.c. of bloodstained fluid, but no frank blood clot.

Pericardial and pleural sacs: Nil abnormal seen.

ALIMENTARY SYSTEM:

Mouth, tongue, oesophagus and stomach: Nil abnormal seen.

1 inch duodenum situated near the neck of the right limb of the pancreas appeared to be gangrenous or at the least not viable. The remainder of the gut showed no abnormality.

The liver was normal superficially and on section.

The common bile duct was patent and undamaged. It entered the second part of the duodenum alone, not being joined by any pancreatic duct.

ENDOCRINE SYSTEM:

Suprarenals: Nil abnormal seen.

Pancreas: 1 inch of pancreas near the head was gangrenous. This part had, during operation, a ligature placed around it and subsequently severed. The remainder of the pancreas showed no abnormality. A little fat necrosis was present in the mesentery around the cut ends.

RETICULO-ENDOTHELIAL SYSTEM)

UROGENITAL SYSTEM

RESPIRATORY SYSTEM

} Nil abnormal seen.

CARDIOVASCULAR SYSTEM:

The right side of the heart was dilated. There was no other abnormality.



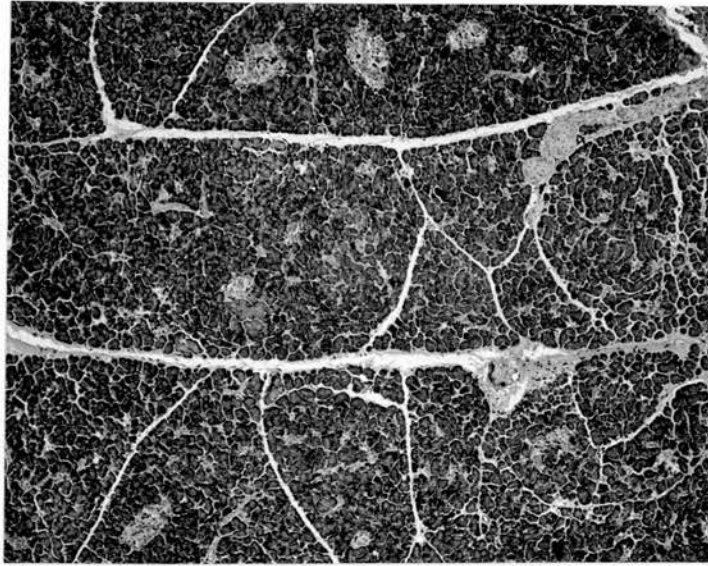


Fig. 47.

Dog 7. Pancreas. Haematoxylin and Eosin.  
 x 45. Body of pancreas with islets  
normal in number and size.

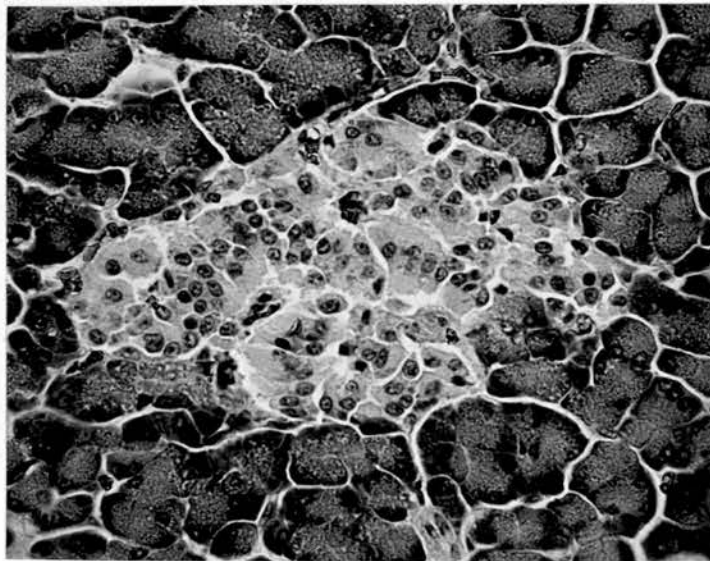


Fig. 48.

Dog 7. Pancreas. Haematoxylin and Eosin.  
 x 350. Higher magnification of a  
 typical normal islet.



Microscopic Report.

Heart and Pericardium: No abnormality is seen.

Lung: shows terminal congestion and oedema only.

Liver: No abnormality seen.

Spleen: Terminal congestion is seen. A good deal of free haemosiderin occurs. Proliferation of the histiocytes and reticulum cells of the sinusoids and pulp has taken place.

Kidney: shows terminal congestion.

Duodenum: The mucosa is exceedingly congested and the epithelium desquamated. Oedema of the subserous fat occurs. The gut at this point, however, may be viable, although the considerable desquamation and pyknosis seen in the villi render this by no means certain.

Pancreas: Some autolysis or digestion was seen at the edges of some lobules and some oedema in the interlobular spaces. The remainder of the section showed no abnormality. (figs. 47 and 48).

Another section of pancreas shows gross oedema and necrosis.

F i n a l   A b s t r a c t .

Death occurred from shock due to prolonged operative trauma.

Gangrene of neck of right limb of pancreas.

Damage to second part of duodenum.

Dog 8.

29.1.53.

Operation. Animal was given 0.3 G. sodium thio-pentone to induce anaesthesia, after which it collapsed and died. Autopsy was not performed. A post-mortem dissection, however, showed that the 2 main pancreatic ducts joined each other before entering duodenum separately from the common bile duct.

10.15 hours. Fasting blood sugar = 96 mgm.%.  
50 mgm. alloxan/Kg. of body weight in 5% solution  
of distilled water was injected intravenously.  
Some leakage outside vein occurred, but this was  
massaged into the tissues very quickly. Approx-  
imately 2 c.c. went outside the vein.

15.35 hours. Blood sugar = 88 mgm.%.  
(Dog limping on leg which had alloxan injection).

11.2.53.

16.35 hours. Fasting blood sugar = 42 mgm.%.  
(Dog still limping on leg which had alloxan).

12.2.53.

12.10 hours. Fasting blood sugar = 92 mgm.%.  
18.2.53.

Right fore-leg shows an abscess, where sodium  
thiopentone was injected.

Right hind-leg - an ulcer has formed, where alloxan  
was injected.

Hot fomentations applied to fore-leg.

20.2.53.

10.30 hours. Fasting blood sugar = 83 mgm.%.  
Urinary sugar (Benedict) = negative.

The dog is clearly not diabetic.

23.2.53.

Weight - 10 Kg.

24.2.53.

14.30 hours. Fasting blood sugar = 74 mgm.%.  
18.2.53.

14.35 hours. 60 mgm. alloxan/Kg. of body weight given intravenously in 5% solution of distilled water.

16.30 hours. Blood sugar - specimen clotted.

27.2.53.

14.00 hours. Fasting blood sugar = 83 mgm.%.

Urinary sugar (Benedict) = negative.

As this dog was not diabetic it was discarded.

Dog 10.

Variety - Collie.

Weight - 11.1 Kg.

5.3.53.

11.00 hours. Fasting blood sugar = 76 mgm.%.

11.05 hours. 60 mgm. alloxan/Kg. of body weight in 5% solution of distilled water given intravenously in less than 60 seconds.

Dog defaecated a few minutes later and looked miserable.

16.00 hours. Blood sugar = 70 mgm.%.

6.3.53.

10.15 hours. Fasting blood sugar = 66 mgm.%.

7.3.53.

10.30 hours. Fasting blood sugar = 126 mgm.%.

10.3.53.

Dog looks rather ill.

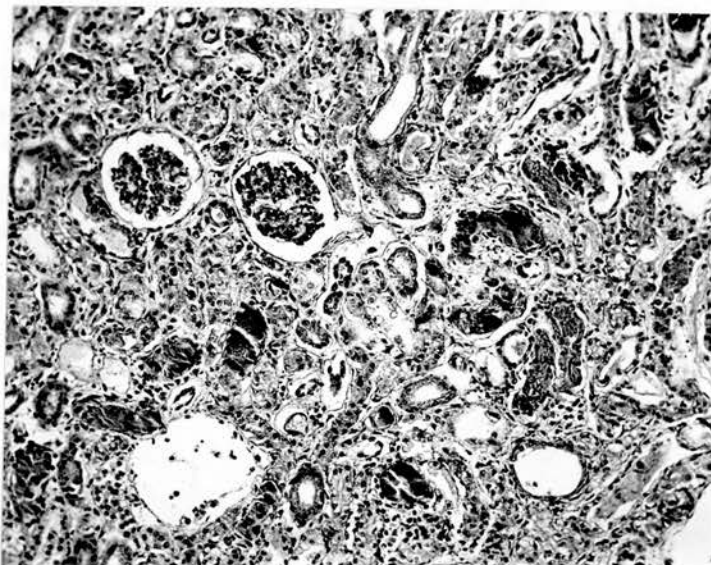


Fig. 49.

Dog 10. Kidney. Haematoxylin and Eosin.  
x 120. Patchy tubular damage due to  
alloxan.

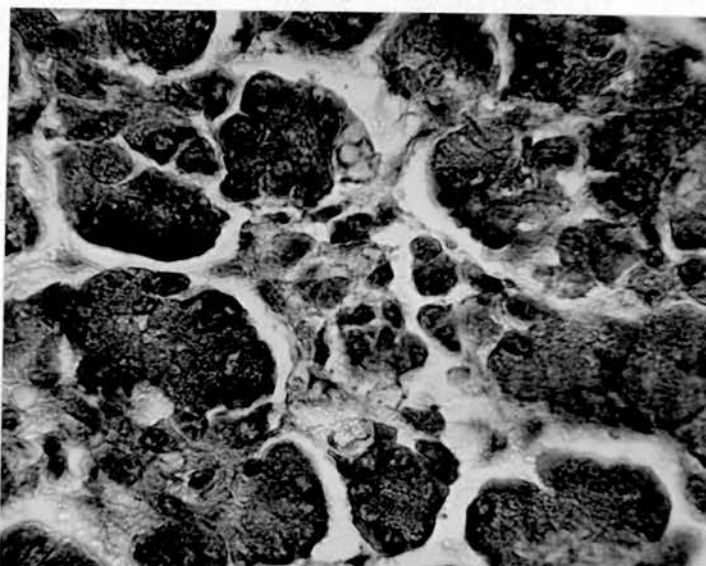


Fig. 50.

Dog 10. Pancreas. Gomori. x 600. Small  
islet of A-cells - alloxan diabetes.

11.3.53.

Still in poor condition. Ketosis ?.

15.00 hours. Fasting blood sugar = 459 mgm.%.

Dog having diarrhoea and is vomiting. Appears very ill.

16.00 hours. Blood urea = 122 mgm.%.

12.3.53.

11.00 hours. Dog died.

Autopsy performed by Dr. B. Cruickshank (A.S.P. indisposed). The kidneys appeared unusually pale - particularly the cortices. Otherwise there was no pathological feature noted.

#### Microscopic Report

Heart: shows no abnormality.

Lung: Marked terminal congestion only is present.

Liver: No abnormality seen.

Spleen: shows terminal congestion. A good deal of haemosiderin occurs, lying free in the pulp.

Kidney: Some patchy necrosis of convoluted tubules is seen. The glomeruli show no abnormality. There is vacuolation of the cells of Henle's loop. The tubules of the latter contain many granular casts. No abnormality of vessels is seen. (fig. 49).

Suprarenal: shows no abnormality.

Pancreas: Haematoxylin and Eosin. The islets are reduced in number and also in size. No other abnormality occurs.

Gomori. The islets appear to consist chiefly of pink-staining cells which, in the main, do not contain visible granules, but which are almost certainly A-cells (fig. 50). A few basophilic ones are also seen and represent B-cells.

Final Abstract.

Death from diabetic-uraemic syndrome produced by alloxan.

Dog 11. Variety - Chow chow.

Weight - 19.5 Kg.

17.3.53.

11.45 hours. Fasting blood sugar = 68 mgm.%.

11.50 hours. 60 mgm. of alloxan/Kg. of body weight given intravenously over 2 minutes in 5% solution of distilled water.

16.15 hours. Blood sugar = 81 mgm.%.

19.3.53.

Dog quite fit. Shows no great thirst.

20.3.53.

14.00 hours. Fasting blood sugar = 91 mgm.%.

24.3.53. Glucose tolerance test.

10.30 hours. Fasting blood sugar = 90 mgm.%.

a meal of 50 G. glucose in saturated solution was given.

11.10 hours. Blood sugar = 122 mgm.%.

12.30 hours. Blood sugar = 122 mgm.%.

13.10 hours. Blood sugar = 105 mgm.%.

26.3.53.

Urinary sugar (Benedict) - negative.

As this dog was clearly not diabetic, it was discarded.



Dog 12.                      Variety - Airedale.  
Weight - 27.7 Kg.

7.5.53.

10.15 hours. Fasting blood sugar = 60 mgm.%.

11.25 hours. 60 mgm. alloxan/Kg. of body weight  
given intravenously in 5% solution of distilled  
water. Injection over 2 minutes.

This dog vomited immediately after injection.

16.10 hours. Blood sugar = 124 mgm.%.

8.5.53.

10.15 hours. Fasting blood sugar = 60 mgm.%.

9.5.53.

10.00 hours. Fasting blood sugar = 431 mgm.%.

10.5.53.

16.00 hours. Dog was vomiting.

17.00 hours. Dog died.

Dog 12.                      Post Mortem - 11.5.53.

At post mortem the right ventricle was  
dilated. The cortex of the kidney was pale and  
soft.

The liver was soft, pale and fatty.

The spleen was soft and rather autolytic.

The pancreas was autolytic. No abnormality  
was found in the other organs.

#### Microscopic Report

Heart: No abnormality is seen.

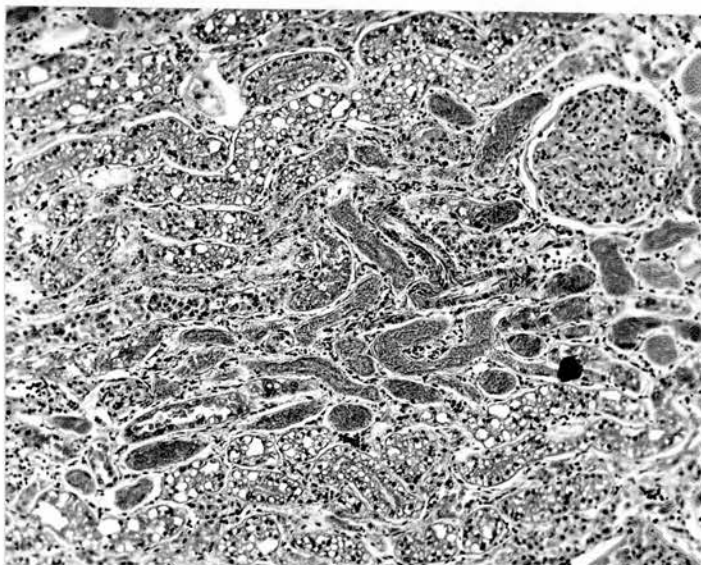


Fig. 51.

Dog 12. Kidney. Haematoxylin and Eosin. x 100. Proximal convoluted tubules show autolysis and distal convoluted tubules extensive fatty vacuolation and some patchy necrosis - alloxan damage.

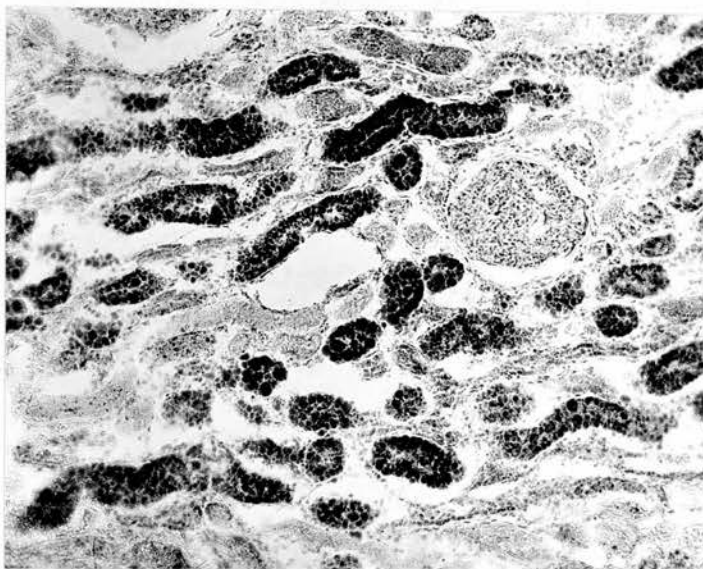


Fig. 52.

Dog 12. Kidney. Scharlach R. x 85. Fatty droplets staining in distal convoluted tubules - alloxan damage.

Lung: A good deal of patchy bronchopneumonia and collapse is present. The exudate in the alveoli consists of polymorphs, macrophages and fibrin.

Kidney: Haematoxylin and Eosin. Some of the proximal convoluted tubules are granular, acidophilic and structureless. This is largely due to autolysis, although there is probably some true damage concealed thus. The distal convoluted tubules show widespread vacuolation. The ascending limb of the loop of Henle shows a less marked fatty degeneration. (fig. 51).

Scharlach R. Fatty change is seen in distal convoluted tubules and ascending limb of Henle's loop. (fig. 52.).

Periodic Acid Schiff: (McManus). The vacuoles in the tubules do not take up the stain.

Spleen: is severely congested. There is no surviving lymphoid tissue around the trabeculae and the pulp consists of red cells and a few histiocytes.

Liver: Haematoxylin and Eosin. Autolysis is advanced. Centri-lobular fatty degeneration is present, but most appearances are obliterated by the post-mortem change and the presence of large numbers of anaerobic bacilli.

Periodic Acid Schiff: Nothing takes up the stain.

Scharlach R. Fatty vacuoles taking up the stain are seen irregularly distributed in the lobules.

Pancreas: Haematoxylin and Eosin. Autolysis is too advanced to identify islets.

Gomori. Autolysis is too advanced to identify islets.

Ovary: No abnormality seen.

### F i n a l     A b s t r a c t .

Death from diabetic-uraemic syndrome due to alloxan.     Patchy bronchopneumonia.

Dog 13.

Variety - Collie.

Weight - 16 Kg.

26.5.53.

10.35 hours. Fasting blood sugar = 85 mgm.%.

10.40 hours. 50 mgm. alloxan/Kg. of body weight given in less than 2 minutes in 5% solution of distilled water.

15.45 hours. Blood sugar = 96 mgm.%.

27.5.53.

10.30 hours. Fasting blood sugar = 101 mgm.%.

28.5.53.

10.15 hours. Fasting blood sugar = 124 mgm.%.

30.5.53.

Urinary sugar (Benedict) - greenish-yellow (+).

Rothera's test for acetone - negative.

1.6.53.

10.15 hours. Fasting blood sugar = 120 mgm.%.

3.6.53.

Urinary sugar (Benedict) - yellow (+).

Rothera's test - negative.

4.6.53.

11.30 hours. Fasting blood sugar = 124 mgm.%.

Animal appears quite bright and cheerful. Not drinking excessive amounts of water.

8.6.53.

Urinary sugar (Benedict) - greenish-yellow (+).

Rothera's test - negative.

Albumen (boiling) - negative.

It is supposed the dog is diabetic.

9.6.53.

10.30 hours. Given 5 units zinc protamine insulin.

14.15 hours.

Operation. Anaesthesia was induced by 0.4 G. sodium thiopentone and open circuit endotracheal ether. The abdomen was opened by a right upper paramedian incision. Pancreas eviscerated and ligatures passed round the neck of right limb, and pancreas divided and cauterized between them. The ligated limb had 6 c.m. black silk threads (double) tied at each end for subsequent identification. Abdomen closed in layers. Operation without incident.

10.6.53.

Recovering from operation.

11.6.53.

Urinary sugar (Benedict) - negative.

12.6.53.

Urinary sugar (Benedict) - negative.

The negative urinary sugars may be due to low food intake on day of operation and subsequently.

**E. E.**

Intake.

9.6.53

NONE

10.6.53

1 pint milk

11.6.53

1 pint milk

12.6.53.

Dog recovering well - wound healthy.

16.6.53.

Urinary sugar (Benedict) - negative.

23.6.53.

Urinary sugar (Benedict) - green turbidity (trace).

25.6.53.

Urinary sugar (Benedict) - greenish-yellow (+).

Rothera's test - negative.

It seems dog is definitely a mild diabetic of permanent type.

27.6.53.

Urinary sugar (Benedict) - greenish-yellow (+).

31.6.53.

Urinary sugar (Benedict) - negative.

Glucose tolerance test.

09.35 hours. Fasting blood sugar = 103 mgm.%.

50 G. of glucose dissolved in water given orally  
without any other food.

10.02 hours - 167 mgm.%.

10.35 hours - 212 mgm.%.

11.10 hours - 260 mgm. %.

11.40 hours - 226 mgm.%.

**12.23 hours - 172 mgm.**

14.10 hours - 120 mgm.%.

It is concluded the dog is mildly diabetic.



1.7.53.

50 mgm. alloxan/Kg. of body weight given intravenously in 5% solution of distilled water in 2 minutes.

6.7.53.

11.00 hours. Fasting blood sugar = 124 mgm.%.

7.7.53.

Urinary sugar (Benedict) - yellow (+).

14.7.53.

Urinary sugar (Benedict) - negative.

This animal is mildly diabetic, but is discarded as having failed to become sufficiently diabetic for the purpose of the experiment.

Dog 14.

Variety - Cairn terrier.

Weight - 5 Kg.

13.7.53.

10.55 hours. Fasting blood sugar = 71 mgm.%.

Dog given intravenously 60 mgm. alloxan/Kg. of body weight in 5% solution of distilled water in 2 minutes.

16.30 hours. Blood sugar = 169 mgm.%.

15.7.53.

12.15 hours. Blood sugar = Specimen clotted.

Urinary sugar (Benedict) - yellow (+).

Blood Urea Nitrogen (B.U.N.) - 33 mgm.%.

16.7.53.

Dog not well. Uraemia ?.

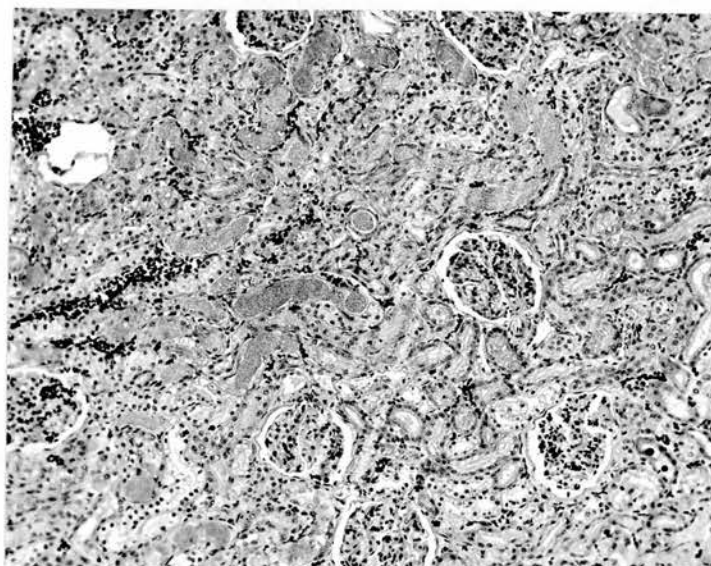


Fig. 53.

Dog 14. Kidney. Haematoxylin and Eosin.  
x 100. Some tubules are necrotic and  
some are filled with granular casts. Fatty  
vacuolation of some distal tubules -  
alloxan damage.

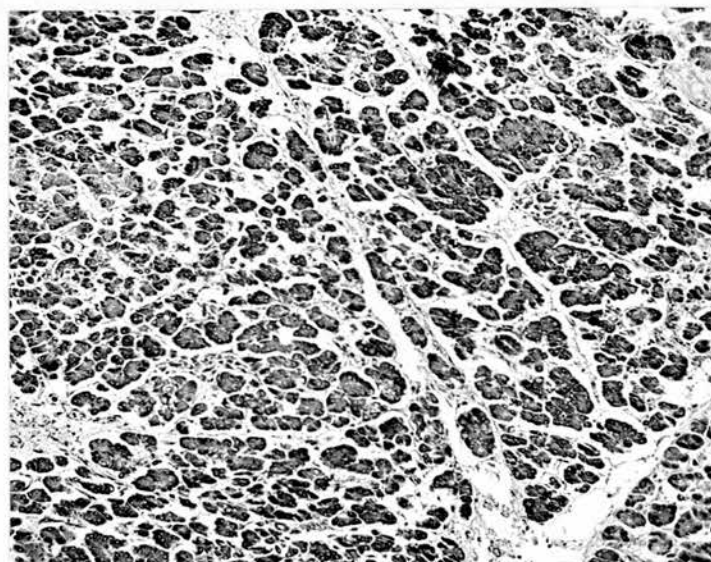


Fig. 54.

Dog 14. Pancreas. Haematoxylin and Eosin.  
x 100. An islet is seen to be reduced in  
size and of crescentic shape - alloxan  
diabetes.

B.U.N. = 33 mgm.%.

Blood sugar = 360 mgm.%.

21.7.53.

Dog died (A.S.P. on holiday). Autopsy by Dr. B. Cruickshank.

Nothing significant seen naked eye at post-mortem examination.

### Microscopic Report.

Heart: Nil abnormal seen.

Lung: Nil abnormal seen.

Liver: A slight degree of fine centri-lobular vacuolation is seen, suggesting fatty degeneration.

Spleen: No abnormality is seen. The free haemosiderin normally present in a dog is noted.

Kidney: Some convoluted tubules, probably distal, are necrotic. This has occurred in a patchy fashion sparing certain nephrons. Tubules of Henle's loop show fatty vacuolation and many contain granular or epithelial casts. The lesions, though severe, do not affect so many nephrons as is sometimes seen in tubular damage due to alloxan. (fig. 53). A section of the other kidney shows a similar appearance.

Suprarenals: Both organs show no microscopic abnormality.

Pancreas: Head - Haematoxylin and Eosin. A few islets are seen reduced in size. (fig. 54). Gomori. The islet cells either do not contain granules or have a reddish tint.

Body - Haematoxylin and Eosin. Many of the islets are reduced in size. Gomori. Islets consist of cells which are the reddish colour of A-cells.

Tail - A similar appearance is noted.

### Final Abstract.

Dog died of diabetic-uraemic syndrome due to alloxan.



Fig. 55.

Dog 16.

Dog 16. Variety - Collie (bitch).

Weight - 11.8 Kg.

26.8.53.

Photographed. (fig. 55).

27.8.53.

Operation. The dog was anaesthetized with sodium thiopentone (0.3 G.) and open circuit ether using endotracheal intubation. An upper abdominal right paramedian incision was made and the pancreas exposed through it. The right limb was divided between black silk ligatures and the cut ends cauterized. A ligature was placed at the end of the right limb to facilitate subsequent identification. The abdomen was closed in layers.

28.8.53.

Condition good.

1.9.53.

Recovered from operation.

14.9.53.

Dog very healthy.

9.10.53.

Weight - 14.1 Kg.

10.45 hours. Fasting blood sugar = 69 mgm.%.

10.50 hours. 60 mgm. of alloxan/Kg. of body weight given intravenously in 5% solution of distilled water.

17.50 hours. Blood sugar = 155 mgm.%.

11.10.53.

11.30 hours. Fasting blood sugar = 132 mgm.%.

12.10.53.

11.00 hours. Fasting blood sugar = 156 mgm.%.

13.10.53.

11.15 hours. Fasting blood sugar = 165 mgm.%.

16.10.53.

10.45 hours. Fasting blood sugar = 134 mgm.%.

Fasting morning urine (Benedict) = greenish  
yellow (+).

Rothera's test for acetone = negative.

10.50 hours. A meal of 50 G. glucose in saturated solution was given orally by forced feeding.

11.40 hours. Blood sugar = 321 mgm.%.

12.45 hours. Blood sugar = specimen insufficient.

14.35 hours. Blood sugar = 370 mgm.%.

15.55 hours. Blood sugar = 217 mgm.%.

(c.f. Graph 3).

It is therefore concluded the dog is moderately DIABETIC.

20.10.53.

Operation. 0.5 G. sodium thiopentone was given intravenously and then endotracheal intubation and ether anaesthesia. Right upper paramedian incision used to expose the pancreas. A small abscess 1 cm. in diameter containing pus was seen



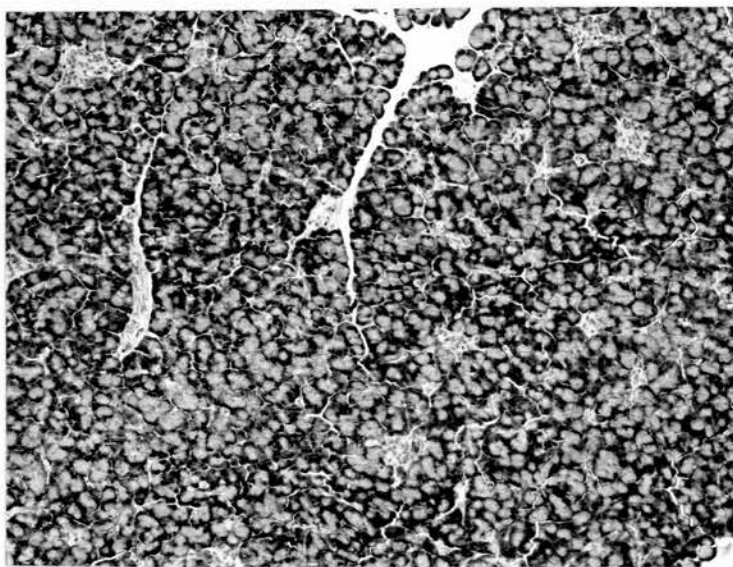


Fig. 56.

Dog 16. Unligated left limb of pancreas. Haematoxylin and Eosin. x 95. The islets are markedly reduced in size and many are collapsed and crescent shaped - alloxan diabetes.

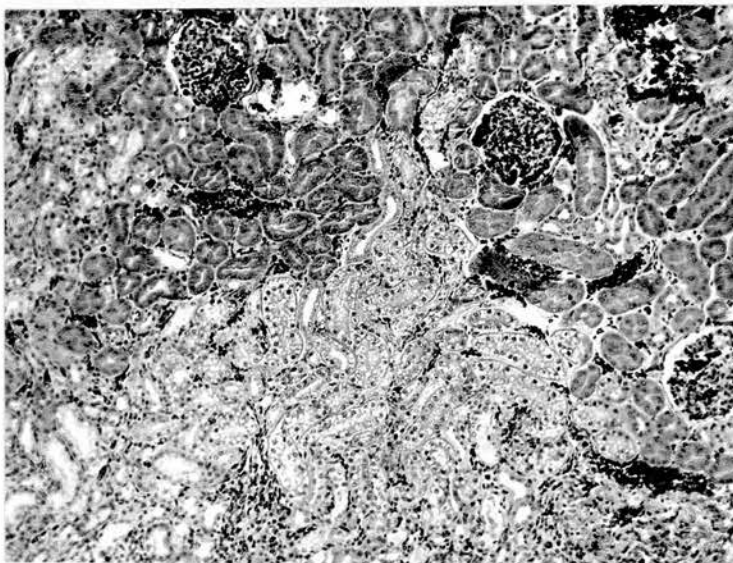


Fig. 57.

Dog 16. Kidney. Haematoxylin and Eosin. x 100. Extensive fatty vacuolation of the convoluted tubules and ascending limb of Henle's loop - alloxan diabetes.

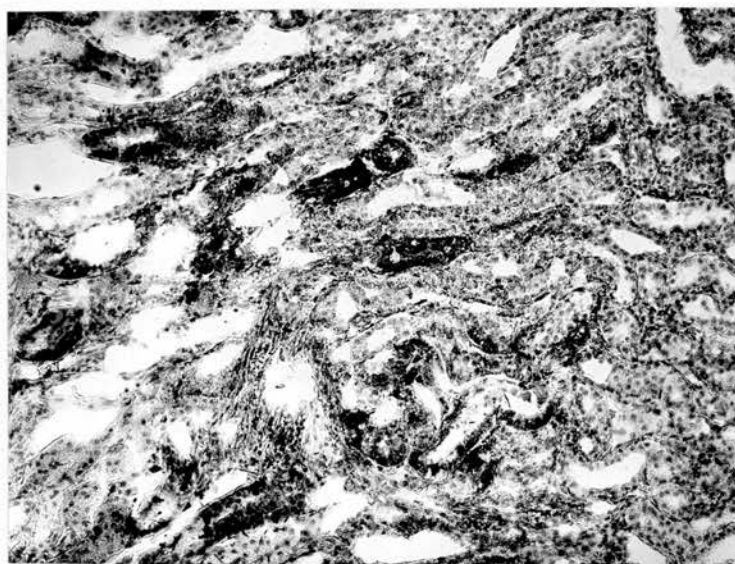


Fig. 58.

Dog 16. Kidney. Scharlach R.  $\times 100$ .  
Fat globules in distal convoluted  
tubules - alloxan diabetes.

at the point of ligation. The left limb was healthy and the right limb was atrophic and moderately infiltrated with fat. It was removed and put into a tube containing sterile Tyrode solution. A further 0.5 G. of sodium thiopentone was given into a mesenteric vein to kill the dog.

Immediately the dog pancreas in suspension was taken to the tissue culture room. The culturing was done as described in Section III, Experiment 2.

Dog 16.                      Post Mortem - 20.10.53.

(P.M. 3 hours after death).

The atrophy of pancreas and the small abscess at the point of ligation were as described in the operation notes. A few adhesions bound coils of small intestine to pancreas and duodenum.

Otherwise no abnormality was seen naked eye and extensive micro was taken and fixed in Zenker. In addition, blocks of pancreas and kidney were fixed in 10% Formol saline.

#### Microscopic Report

Pancreas (unligated left limb) - Haematoxylin and Eosin (5 blocks). Fixed 5 minutes after death. The islets are much reduced in size and many are crescent shaped. There is otherwise no abnormality to be seen. It is noted, however, that the formol fixed preparations are less well preserved than the Zenker fixed ones taken at the same time (fig. 56).

Gomori. The cells of the islets show a marked predominance of A-cells containing red granules (fig. 33). In a few islets hyaline bands are seen between the cells. The islets are reduced in size as in the Haematoxylin and Eosin section.

Pancreas (ligated). Haematoxylin and Eosin: Section shows much fibrosis and round cell infiltration around a main duct and around smaller ductules. There is almost complete atrophy of the parenchymatous exocrine tissue and replacement by fibrous and oedematous chronic granulation tissue. (fig. 31). Some haemosiderin is seen, probably resulting from haemorrhage at the time of ligation. Islets are hard to identify amongst the atrophic acini, but some are made out. They are invested and penetrated by fibrous tissue.

Gomori. The islets examined are seen to consist of A-cells containing red granules. (fig. 32).

Heart: No abnormality is seen.

Lung: No abnormality is seen.

Liver: No abnormality is seen.

Spleen: The organ is moderately congested and the usual amount of haemosiderin seen in the dog spleen is noted. Some proliferation of the reticulum cells at the centre of the Malpighian bodies occurs.

Kidney: Haematoxylin and Eosin. (fixed 5 minutes after death).

Cloudy swelling is seen in the proximal convoluted tubules. There is fatty degeneration in the distal convoluted tubules and in the ascending limb of Henle's loop. (fig. 57).

Scharlach R. A section stained in this way for fat shows heavy fatty deposits in the cells of the distal convoluted tubules and in the ascending limb of Henle's loop. (fig. 58).

Suprarenal Gland: No abnormality is seen. The medulla appears to be almost entirely chromaffin.

Thyroid and parathyroid gland: In some areas the thyroid vesicles are large and contain thick colloid. Elsewhere the colloid is thin and the vesicles small, but there is nothing else noted. The parathyroid shows no abnormality.

Wall of abscess (at site of ligature): Shows an abscess wall composed of fibrous granulation tissue which is arranged around the abscess cavity. Collections of polymorphs are seen in it here and there. The abscess wall lies adjacent to duodenum.

Final Abstract.

Alloxan diabetes.

Successful ligation of right limb of pancreas.  
Small abscess near ligature.

Extensive fatty degeneration of distal tubules.



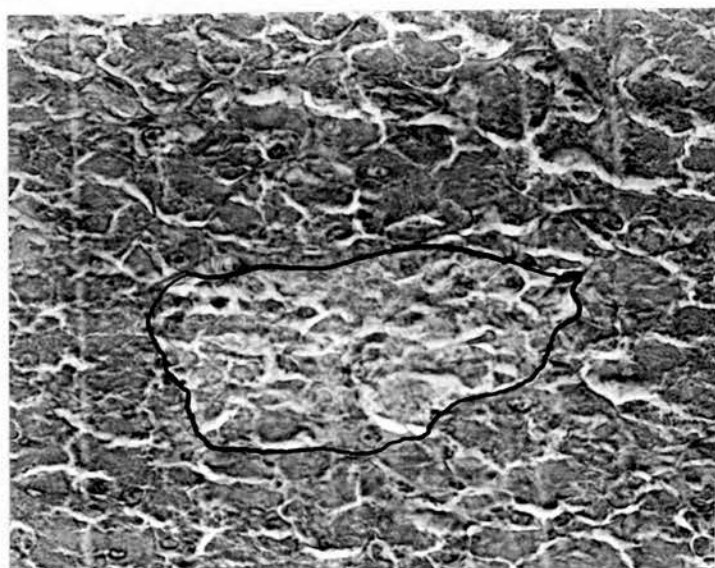


Fig. 59.

Rat 25. Pancreas. Gomori. x 425.  
Islet consisting entirely of A-cells.

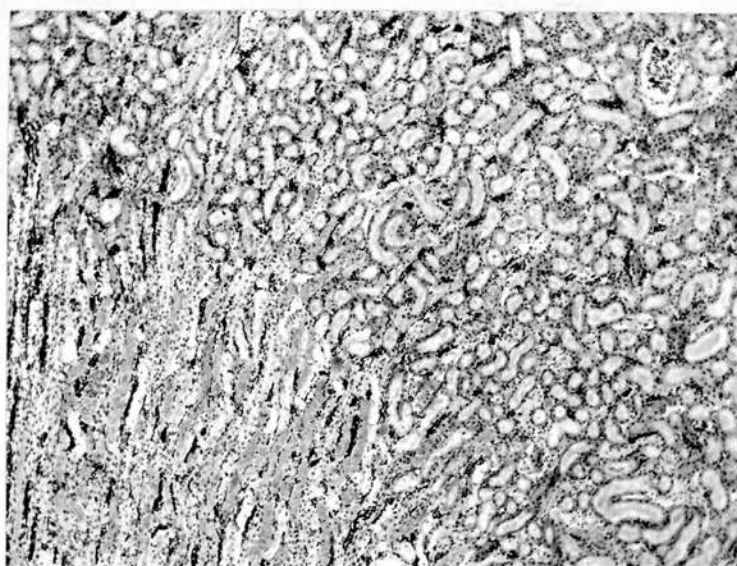


Fig. 60.

Rat 30. Kidney. Haematoxylin and Eosin.  
x 65. Damage to distal tubules with  
formation of granular casts - result of  
alloxan.



APPENDIX 11 b. (c.f. Section IV).

Histological appearances observed in a random selection of rats used in the attempted preparation of alloxan diabetic, hypophysectomized adrenalectomized (A.D.H.A.) rats.

Rat 25: Urinary sugar (Benedict) = negative.

Lung: No abnormality is seen.

Liver: No abnormality is seen except a mild degree of centrilobular fatty degeneration.

Kidney: Coagulative necrosis is seen in the distal convoluted tubules. The proximal convoluted tubules are unaffected.

Pancreas: Haematoxylin and Eosin - Some of the few islets seen are healthy, but others are reduced in size.

Gomori - Islets are seen showing central necrosis and a surviving rim of A-cells. A few islets show A-cells surviving in the centre of an islet, but generally they are situated near the margin. (fig. 59).

Death from uraemia.

Rat 30: Urinary sugar (Benedict) = orange (++).

Kidney: The ascending limb of Henle's loop and distal convoluted tubules in many nephrons show

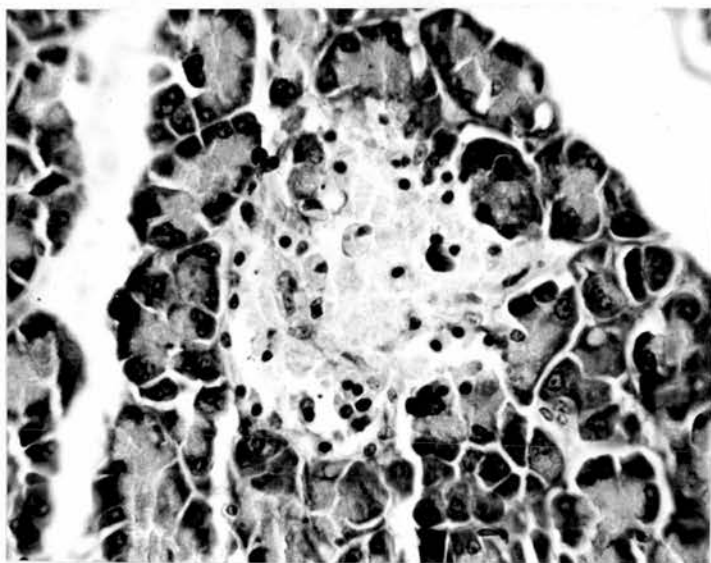


Fig. 61.

Rat 30. Pancreas. Haematoxylin and Eosin.  
x 425. Central necrosis of islet -  
alloxan diabetes.

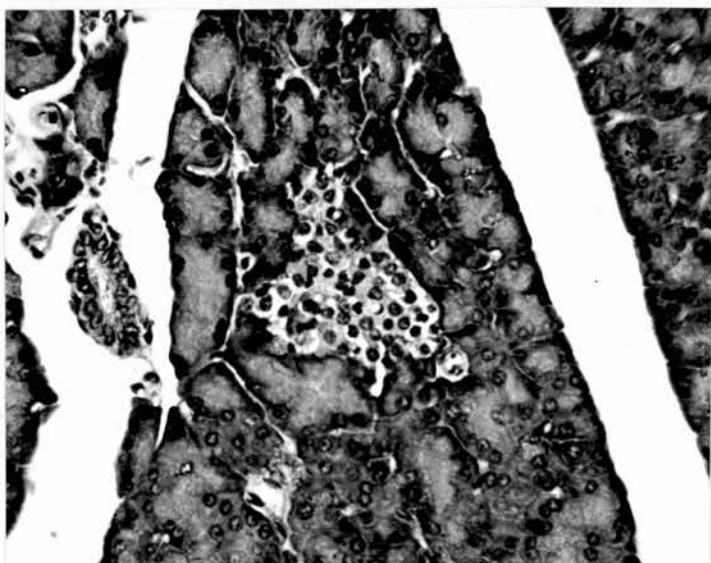


Fig. 62.

Rat 59. Pancreas. Haematoxylin and Eosin.  
x 375. Pyknosis in centrally situated  
B-cells; A-cells unaffected. Lateral  
budding of islet indicating regeneration  
- alloxan diabetes.

damage and contain granular casts. (fig. 60).  
The collecting tubules show fatty degeneration.  
The proximal convoluted tubules are relatively unaffected.

Pancreas: Haematoxylin and Eosin.- Many islets show central necrosis (fig. 61) and in many others complete destruction is noted. Residual cells are seen at the periphery of the islets.

Gomori - It is seen the surviving cells are chiefly A-cells and the B-cells are necrotic. The A-cells are at the periphery of the islets.

Lung: No abnormality is seen.

Death from diabetic-uraemic syndrome.

Rat 31: Urinary sugar (Benedict) = red (+++).

Kidney: Slight granularity of the convoluted tubules is the only abnormality seen.

Pancreas: Haematoxylin and Eosin - Some aggregations of polymorphs are seen. The islets appear to be reduced in size.

Gomori - The pancreas is slightly autolytic, and it is difficult to distinguish the islet cells clearly, but where this can be done the majority appear to be staining red as A-cells.

Lung: Nil abnormal seen.

Liver: A moderate amount of centrilobular atrophy is present.

Death from diabetes.

Rat 34: Urinary sugar (Benedict) = yellow (+).

Liver: No abnormality is seen.

Kidney: Patchy fatty degeneration and extensive necrosis of distal convoluted tubules and ascending limb of Henle's loop.

Lung: No abnormality is seen.

Pancreas: Haematoxylin and Eosin - The islets are of more or less normal size and shape.

Gomori - Both A and B-cells are seen in the islets, although there are some necrotic cells seen, which are probably B-cells.

Death from diabetic-uraemic syndrome.

Rat 40: Urinary sugar (Benedict) = negative.

Kidney: The distal convoluted tubules and ascending limb of Henle's loop show severe coagulative necrosis. The proximal convoluted tubules are unaffected.

Pancreas: Haematoxylin and Eosin - The islets

are apparently unaffected by the alloxan. Some interstitial polymorphonuclear infiltration is seen.

Gomori - Islets appear to be unaffected.  
A and B-cells are seen.

Spleen: No abnormality seen.

Liver: No abnormality seen.

Lung: No abnormality seen.

Heart: No abnormality seen.

Death from uraemia.

Rat 46: Urinary sugar (Benedict) = red (+++).

Pancreas: Haematoxylin and Eosin - The islets are reduced in size and have a distorted shape. Some show central necrosis.

Gomori - A-cells are seen to have survived.

Kidney: No abnormality is found.

Heart: No abnormality is found.

Lung: No abnormality is found.

Liver: Is a little autolytic, but is otherwise normal.

Death from diabetes.

Rat 59: Urinary sugar (Benedict) = red (+++).

Heart: No abnormality seen.

Liver: No abnormality seen.

Spleen: No abnormality seen.

Kidney: No abnormality seen.

Pancreas: Haematoxylin and Eosin - The islets are much reduced in size and under high power it is seen that some of the islet cells are pyknotic. (fig. 62).

Gomori - The surviving cells in the islets are seen to be almost entirely A-cells.

Death from insulin convulsions in a diabetic animal.

Rat 64: Urinary sugar (Benedict) = yellow (+).

Pancreas: Haematoxylin and Eosin - It is seen that islet cells remain only around the periphery of the islets and that the centres consist of pyknotic cells and histiocytes. Some vacuolated cells without nuclei are also seen. (fig. 63).

Gomori - The islets are seen to consist almost entirely of cells with red granules in the cytoplasm and only an occasional surviving blue-staining B-cell.



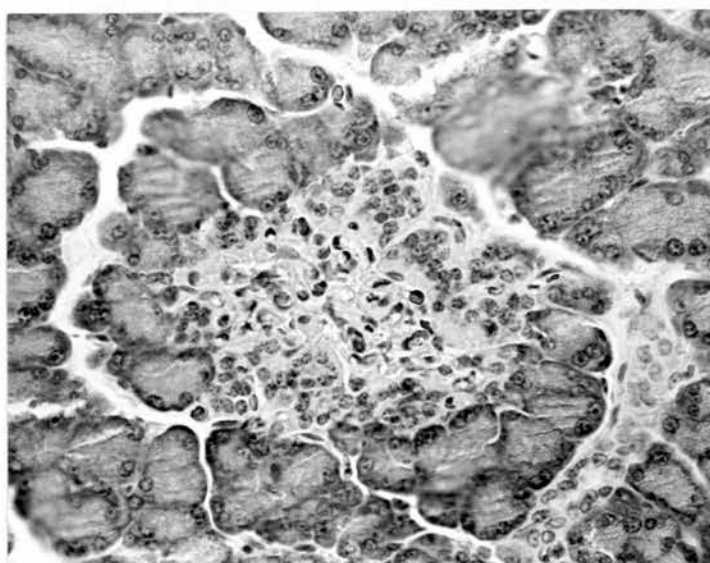


Fig.63.

Rat 64. Pancreas. Haematoxylin and Eosin.  
x 480. Marked pyknosis of central B-cells.  
Peripheral A-cells unaffected - alloxan  
diabetes.

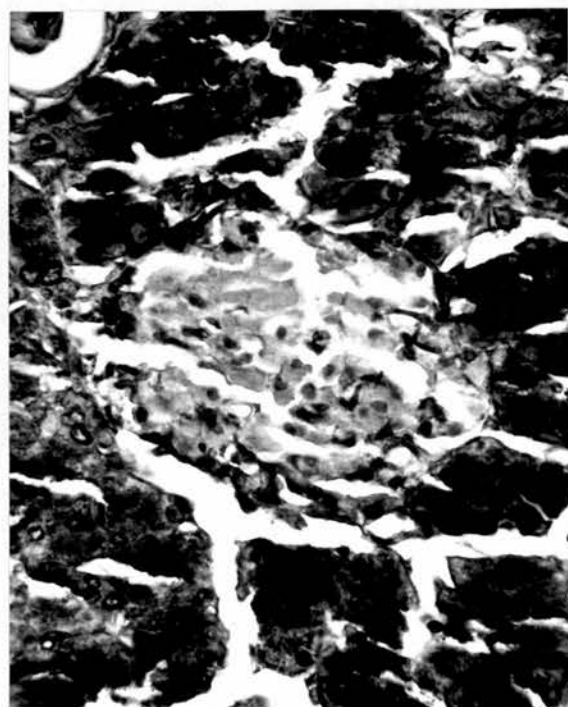


Fig. 64.

Rat 68. Pancreas. Gomori. Partial  
necrosis of islet with only a few A-cells  
remaining.

Heart: No abnormality is found.

Lung: Small areas of vesicular emphysema are seen, but no other feature.

Liver: No abnormality is seen.

Spleen: Is moderately congested. Haemosiderin is present in the pulp. Otherwise there is no abnormality.

Kidney: Some little fatty vacuolation of the cells of the ascending limb of Henle's loop and in the distal convoluted tubules is seen. This is a minor feature, and there is no other kidney damage.

Death from diabetes.

Rat 68: Urinary sugar (Benedict) = yellow (+).

Heart: No abnormality is seen.

Lung: No abnormality is found.

Liver: No abnormality is found.

Spleen: No abnormality is found.

Kidney: Autolysis is moderately far advanced, but no lesion can be seen to suggest tubular damage.

Pancreas: Haematoxylin and Eosin - The islets are

small and reduced in number.

Gomori - The small islets seen consist almost entirely of A-cells with red granules. In one islet some blue-staining necrotic debris is seen, probably derived from B-cells. (fig. 64).

Death from diabetes.

Rat 71: Urinary sugar (Benedict) = orange (++).

Heart: No abnormality is seen.

Lung: No abnormality is seen.

Liver: No abnormality is seen.

Spleen: The organ is markedly congested and the Malpighian bodies show moderately reactive centres. Otherwise there is no abnormality.

Kidney: No abnormality is seen.

Pancreas: Haematoxylin and Eosin - On the whole the islets are not reduced in size, but many appear disarticulated and their nuclei pyknotic.

Gomori - Some islets are seen of normal size, consisting chiefly of A-cells with occasional pyknotic B-cells amongst them.

Death from insulin convulsions in a diabetic animal.

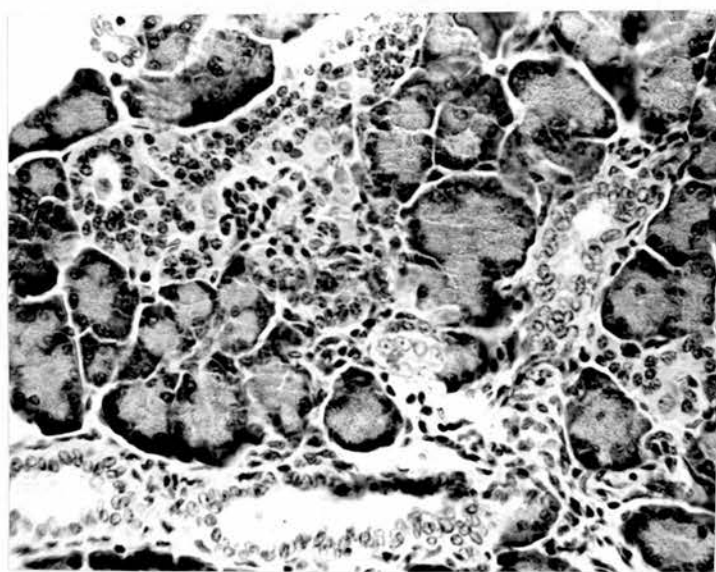


Fig. 65.

Rat 81. Pancreas. Haematoxylin and Eosin.  
x 350. Islets regenerating from ductules.  
(9 days after alloxan administration).

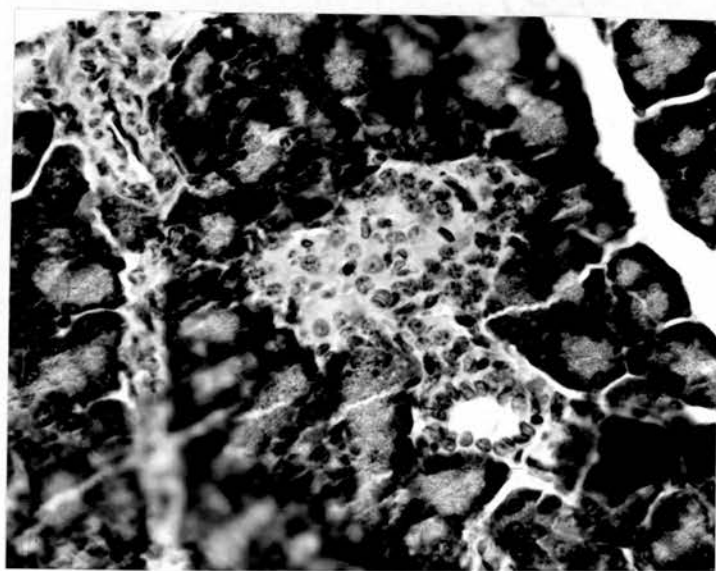


Fig. 66.

Rat 85. Pancreas. Haematoxylin and Eosin.  
x 480. Islet regenerating from a ductule.  
(9 days after alloxan administration).

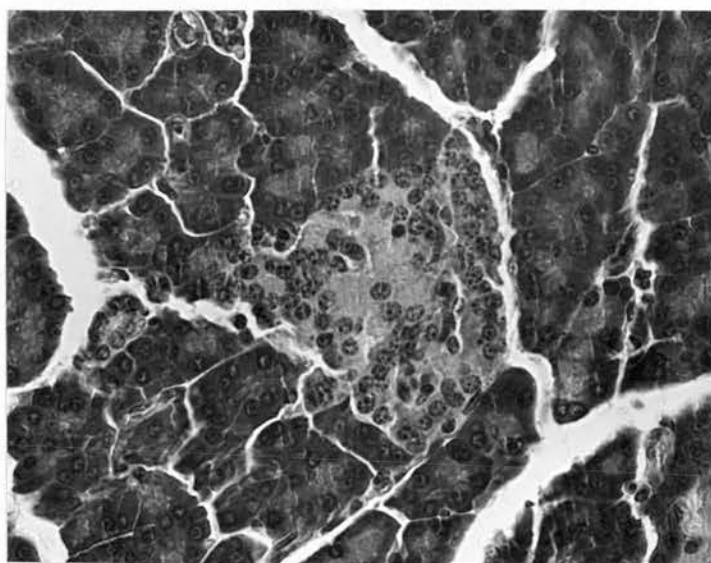


Fig. 67.

Rat 85. Pancreas. Gomori. x 425.  
Decreased number of cells in centre  
of islet. Peripheral ones are A-cells  
- alloxan diabetes.

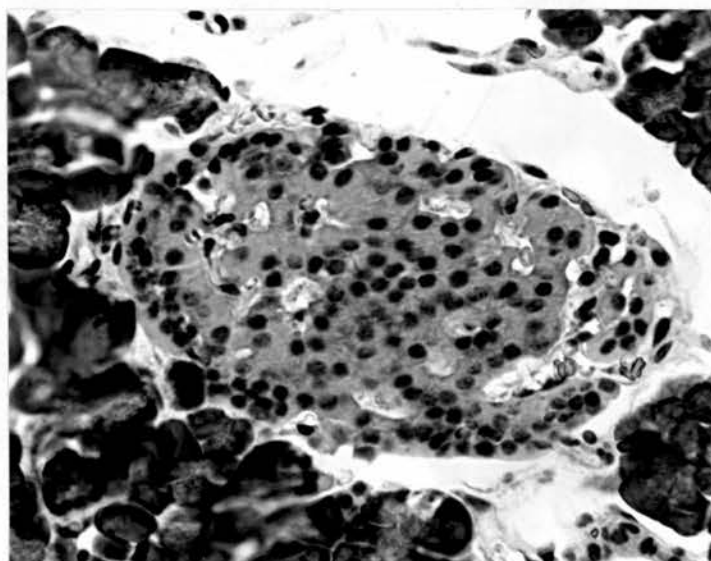


Fig. 68.

Rat 108. (Normal). Pancreas. Haematoxylin  
and Eosin. x 480. Normal rat islet.

Rat 75: Urinary sugar (Benedict) = red (+++).

Heart: No abnormality is seen.

Lung: No abnormality is seen.

Liver: No abnormality is seen.

Kidney: No evidence of tubular damage.

Pancreas: Haematoxylin and Eosin - Shows central necrosis of islets and reduction in number of islets seen.

Gomori - The surviving islet cells are A-cells almost entirely.

Death from diabetes.

Rat 81: Urinary sugar (Benedict) = orange (++).

Heart: No abnormality is seen.

Lung: Some patchy collapse is seen.

Liver: No abnormality is seen.

Kidney: No abnormality observed; in particular, no tubular damage, such as alloxan causes.

Spleen: No abnormality is seen.

Pancreas: Haematoxylin and Eosin - In general there is no marked reduction in the size or number of the islets in this section, although their shape is often distorted. In some places islets appear to be present in very close proximity to ductules suggesting they may have regenerated from them. (fig. 65).



Gomori -The majority of the remaining islet-cells take a reddish tinge in their cytoplasm, although true granules are not present.

A section of sella turcica and sphenoid sinus shows fragments of bone, cartilage, brain and upper respiratory mucosa, but no evidence of pituitary.

Diabetic rat - died after hypophysectomy.

Evidence of islet regeneration from ductules.

Rat 84: Urinary sugar (Benedict) = not done.

(Died within 24 hours of alloxan).

Heart: No abnormality is seen.

Lung: No abnormality is seen.

Liver: No abnormality is seen.

Kidney: No abnormality. No alloxan damage evident.

Pancreas: Haematoxylin and Eosin - Some pyknosis is seen at the centre of the islets. In the interlobular planes is an infiltration by acidophil fluid containing polymorphs and macrophages. Part of the section shows autolysis.

Gomori - There are not many islets on the section. Those which are seen appear to be disarticulated and some of the cells are necrotic. It is not possible to distinguish whether the

surviving islet cells are of A or B type.

It is most likely, in view of the early onset, that death was due to hypoglycaemia following alloxan.

Rat 85: Urinary sugar (Benedict) = yellow (+).

Heart: No abnormality is seen.

Lung: No abnormality is seen.

Liver: No abnormality is seen.

Spleen: No abnormality is seen.

Kidney: No abnormality is seen; in particular, there is no evidence of tubular damage.

Pancreas: Haematoxylin and Eosin - The centres of some islets contain hyaline material and very few nuclei. The latter are generally situated around the periphery of the islets. Many islets are greatly reduced in size. In places islets are seen regenerating from the ductules. Some show budding as well. (fig. 66).

Gomori - Small islets are seen with a rim of cells at the periphery staining red as A-cells. (fig. 67). Many of the cells of the regenerating islets are bluish, and are probably B-cells, but do not contain very definite granules.

Thymus: As far as can be recognised, there is no pathological lesion present.

Diabetic rat - Died under anaesthesia during hypophysectomy.

Evidence of islet regeneration from ductules.

Rat 90: Urinary sugar (Benedict) = yellow (+).

Heart: No abnormality is seen.

Lung: No abnormality is seen.

Kidney: No abnormality is seen; in particular, there is no evidence of tubular damage.

Spleen: Is congested, but otherwise normal.

Liver: No abnormality is seen.

Pancreas: Haematoxylin and Eosin - Numerous sections have been taken, but there are few islets present on them. Those which are seen appear to be of normal shape and size.

Gomori - Islets are seen to consist almost entirely of red-staining A-cells, with only an occasional B-cell seen.

This alloxan diabetic hypophysectomized rat died 6 days after hypophysectomy as a result of endocrine imbalance.

Rat 98: Urinary sugar (Benedict) = orange (++).

Heart: No abnormality is seen.

Lung: No abnormality is seen.

Liver: No abnormality is seen.

Spleen: No abnormality is seen.

Kidney: Autolysis fairly marked, but if tubular damage had occurred, it was probably not very gross.

Pancreas: Autolysis makes description useless.

Diabetic rat - Died after hypophysectomy.

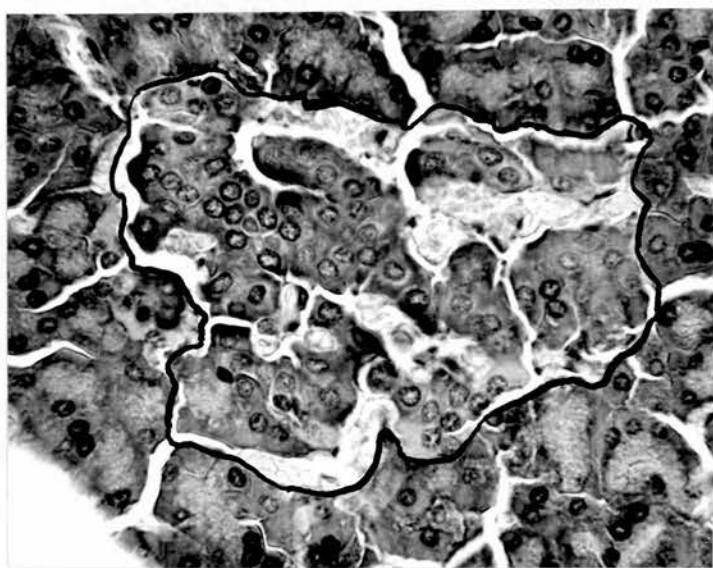


Fig. 69.

Rabbit 4. Pancreas. Gomori. x 575.  
Islets composed of intertwining  
trabeculae. Dark B-cells are seen  
centrally and light A-cells at the  
edges of the trabeculae.

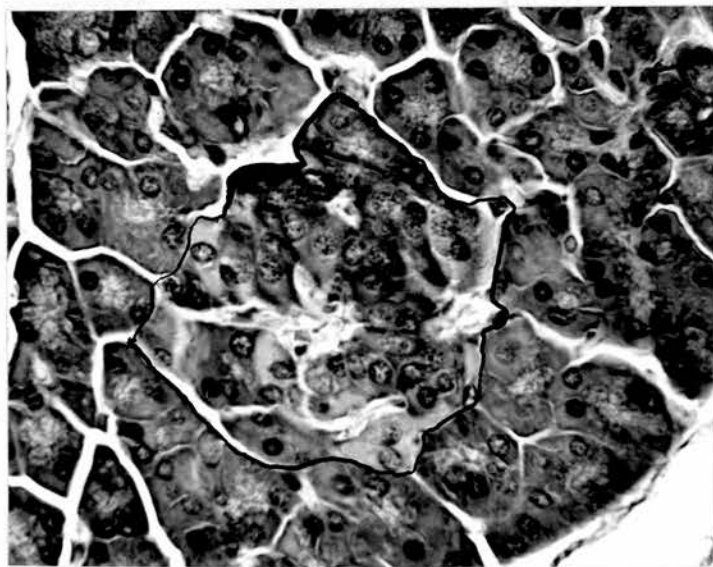


Fig. 70.

Rabbit 4. Pancreas. Gomori. x 575.  
Similar arrangement of A and B-cells  
to that in Fig. 69. The trabecular  
structure is less obvious.

APPENDIX II c. (c.f. Section III).  
(and Appendix iii).

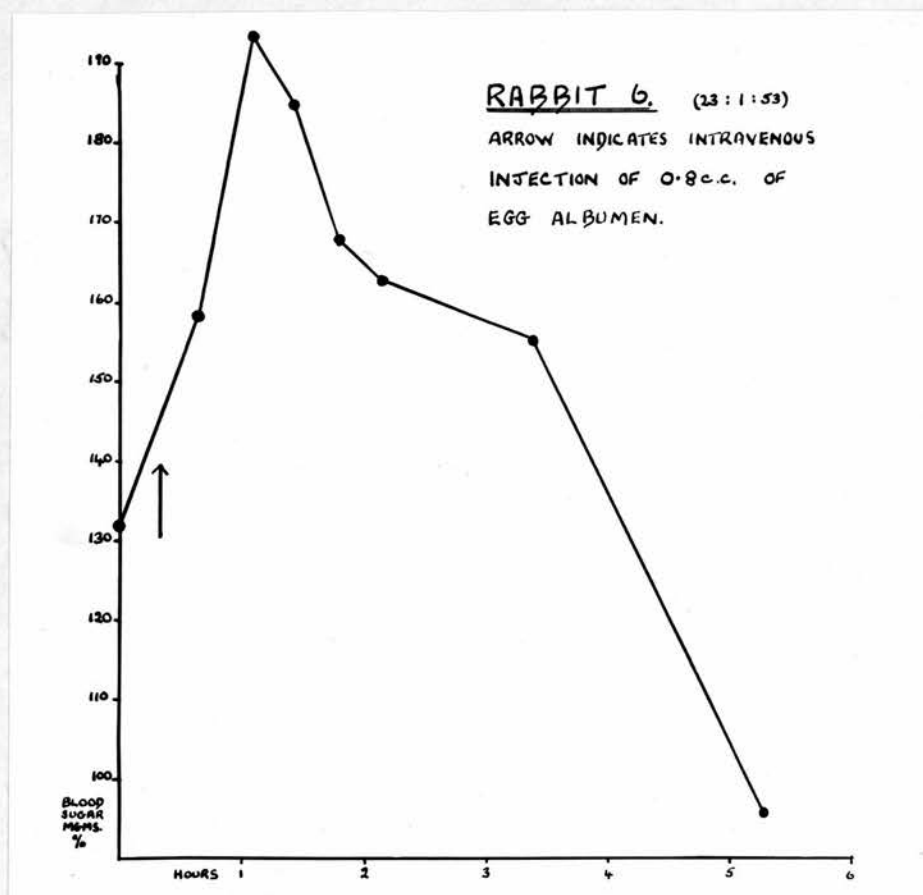
Autopsy findings in Rabbits.

Autopsy was performed on all the rabbits used for the assay of the supernatant fluids of the cultures in Section III, Experiment 1, and in the investigation of the effects of various proteins recorded in Appendix iii. The rabbits used to assay the extracts of supernatant fluids from the cultures in Section III, Experiment 2 were also autopsied. It is not proposed to include these rather lengthy reports as little of significance was found except that Rabbits 2, 3, 5 and 7 showed a pyogenic pyelonephritis. These rabbits were all ones used in Experiment 1 and in the work on the hyperglycaemic effect of proteins. Thus they all had been injected with varying amounts and types of sera. The number of rabbits so affected is, however, too small to draw any conclusions as to whether the serum injections were the causative factor of the pyogenic pyelonephritis.

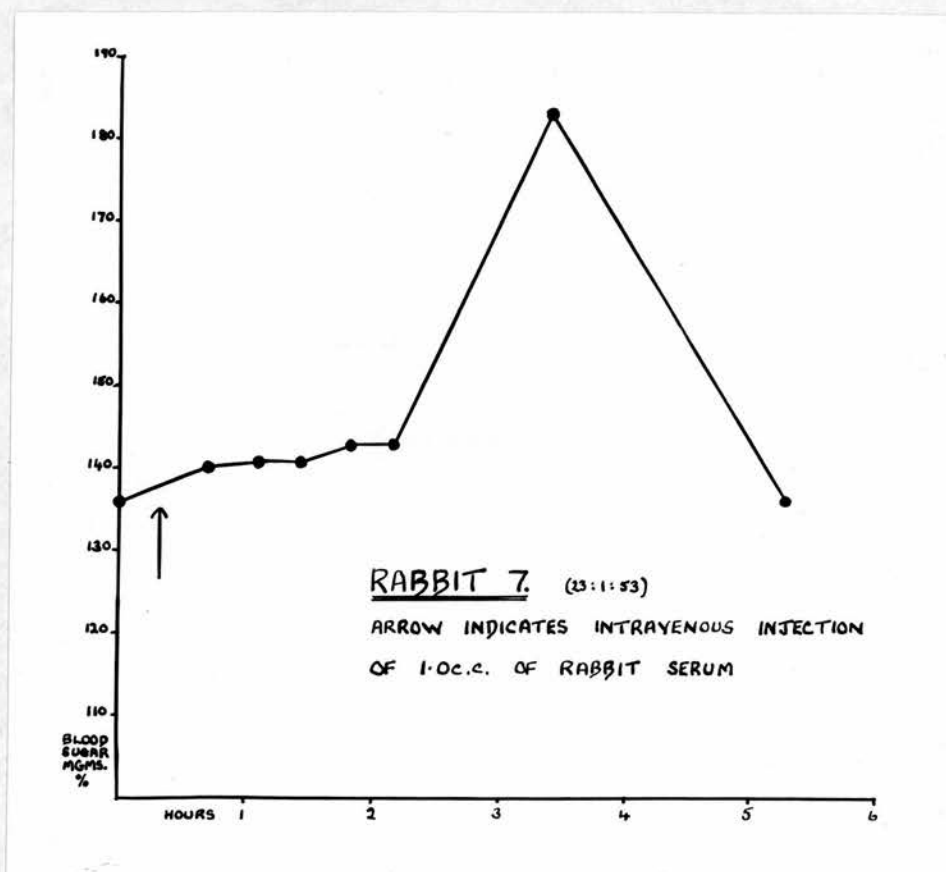
The pancreatic islets of the rabbit were composed of intertwining trabeculae consisting of A-cells and B-cells. Capillaries lie between the trabeculae. The A-cells are situated at the edges of the cords and the B-cells at the centre. (figs. 69 and 70). Because of this arrangement the A-cells are not so frequently at the periphery of the islet as in dog or rat. The kidneys and the



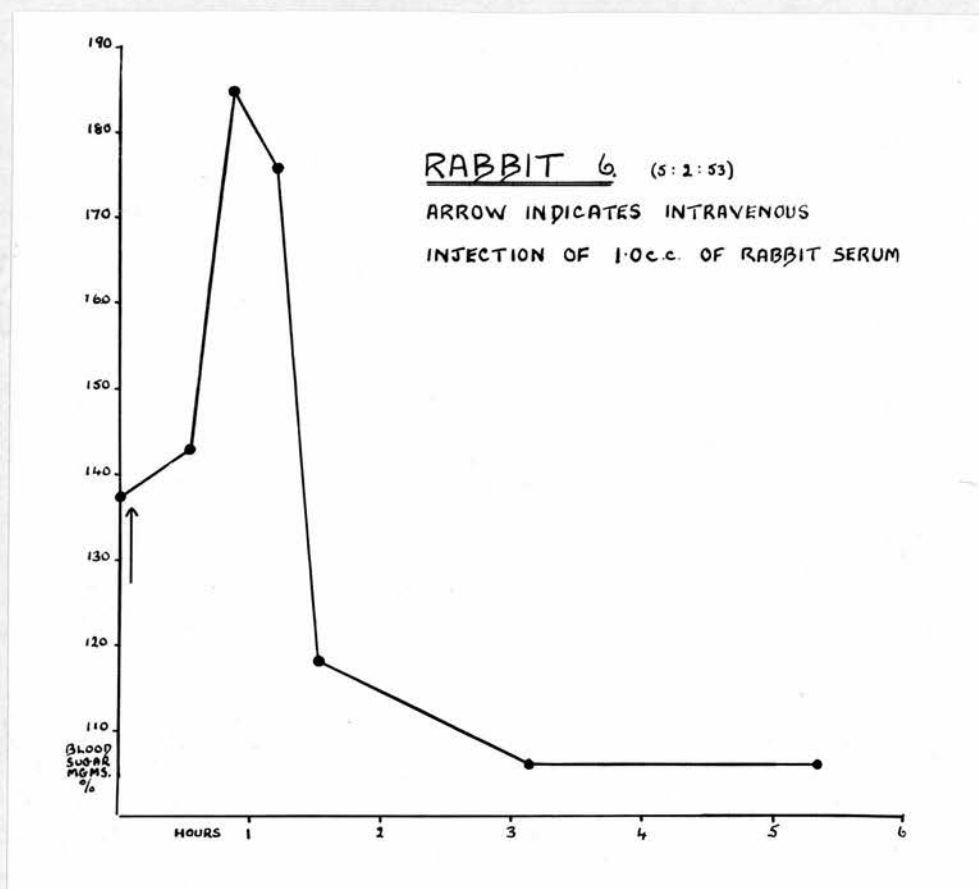
arteries were particularly examined for any evidence of arteritis or glomerulo-nephritis which might have resulted from the injections of serum, but no such lesions could be found although careful examination was made of an extensive series of blocks in each rabbit, taken from heart, lung, liver, kidneys, suprarenals, ovary, pancreas and stomach.



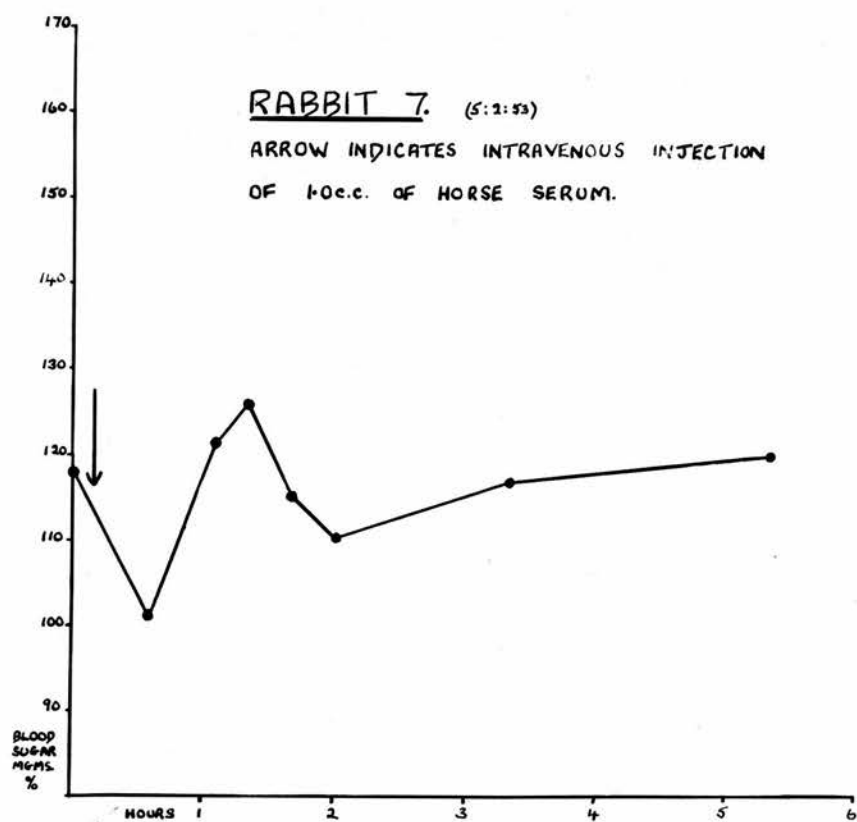
Graph 30.



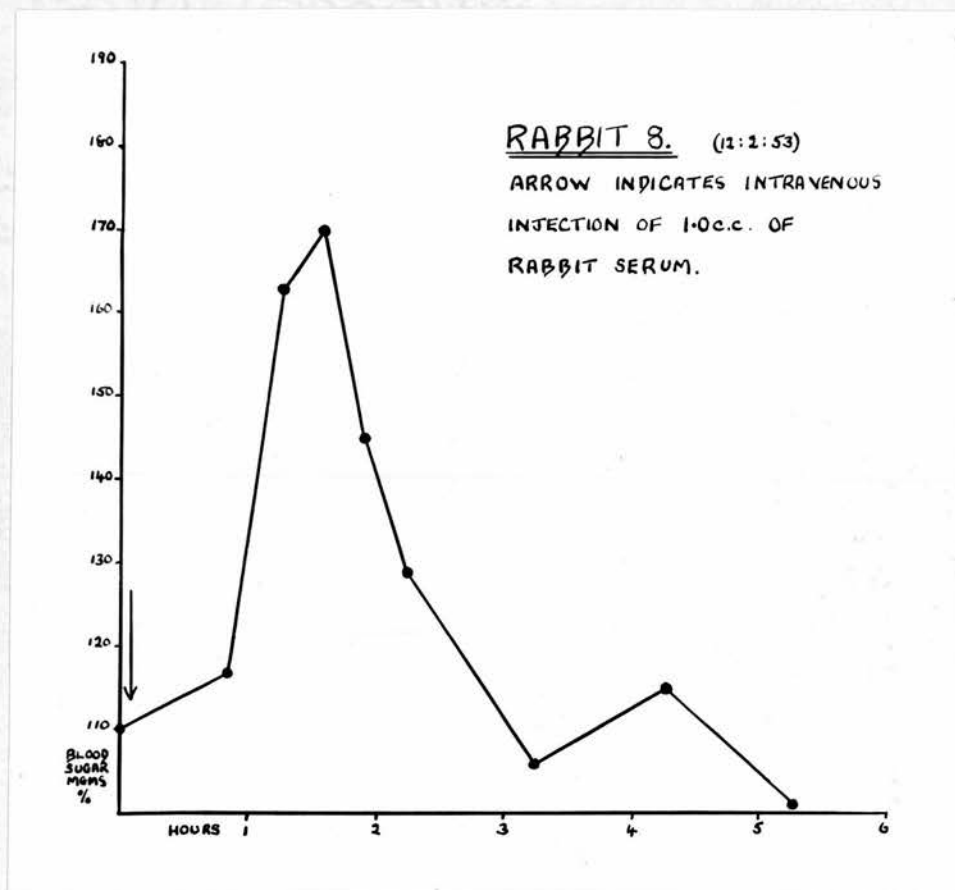
Graph 31.



Graph 32.

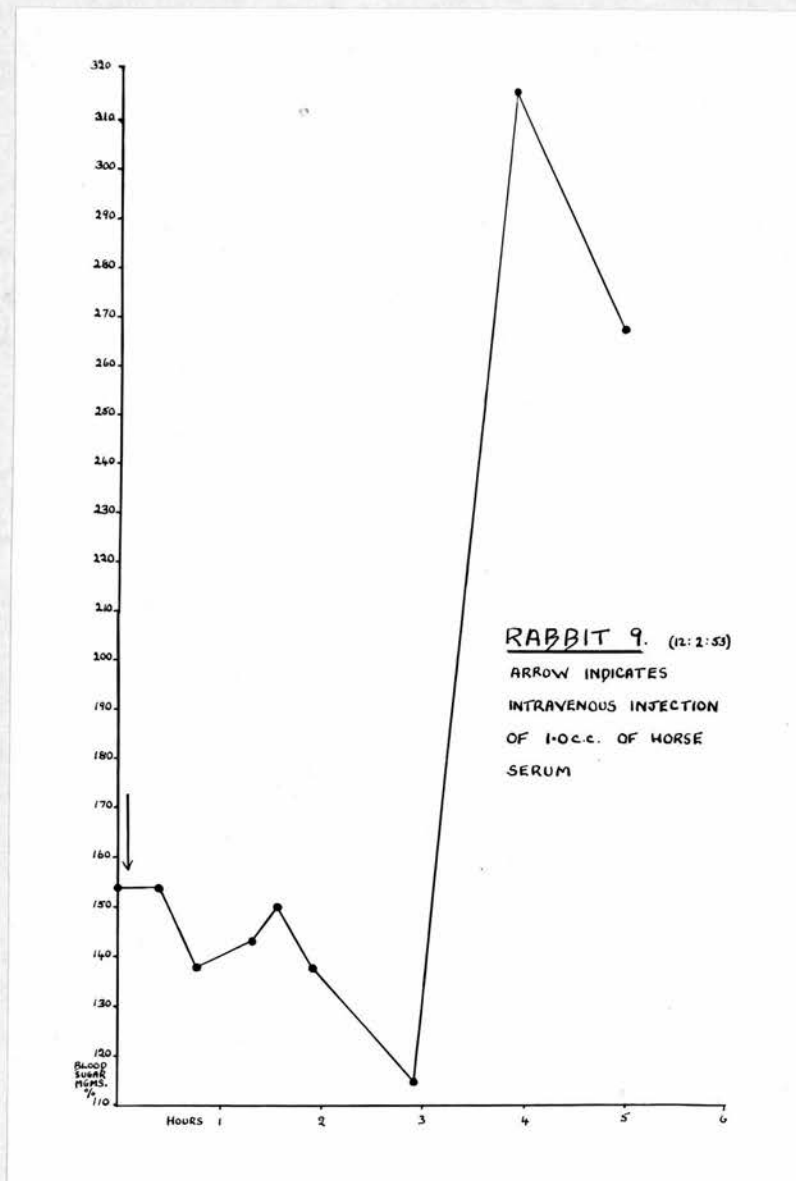


Graph 33.



Graph 34.





Graph 35.

APPENDIX iii. (c.f. Sections I and III).Effects of various proteins injected intravenously into rabbits.

In Section III, Experiment 1, of this thesis it was noted that the injection of the reagent control supernatant fluids, and more specifically, the human serum of which they were largely composed, would cause a marked hyperglycaemic response in rabbits.

It was thought of some interest, therefore, to find out if only human serum would produce this effect, or if sera of other animals, and other proteins would do it also. In short, (1) whether it was a specific property of human serum; (2) If it was not specifically caused by human serum, but could be caused by other sera, whether it was due to some contained factor in the sera, or whether it was a property of protein.

Graph 30 shows that egg albumen can produce a very marked hyperglycaemic reaction. It would seem, therefore, that protein can produce hyperglycaemia in rabbits, and that it is not necessarily due to a contained factor in animal serum.

Graph 31 shows, rather unexpectedly, that homologous rabbit serum can produce a hyperglycaemic response, although it is delayed  $2\frac{1}{2}$  hours after

injection of the serum. A similar though more immediate response is seen in Graphs 32 and 34. These graphs (31, 32 and 34) were recorded on 3 different rabbits.

Graphs 33 and 35 illustrate the effect of horse serum on rabbits which is marked in one instance although insignificant in the other. As these serum effects were not really relevant to the problem under investigation they have not been pursued further meantime, and are recorded here out of interest.

It is understandable that this hyperglycaemic effect of protein might be produced when foreign proteins are injected, e.g. egg albumen, horse and human serum, but even here the explanation is not known, although it is possible that some kind of stress reaction takes place with liberation of adrenaline and glycogenolysis. It is much more difficult, however, to see why such a reaction should be invoked by homologous rabbit serum, and no explanation can at the moment be offered.

#### Summary

(1) Various proteins including horse and rabbit serum and egg albumen were injected intravenously into rabbits.

(2) Hyperglycaemia usually results from such an injection.

(3) This hyperglycaemia is thought to be a property of protein.

(4) No explanation of this is offered, and the hyperglycaemic effect of homologous rabbit serum is particularly puzzling.

(5) It is emphasized that these results were obtained in passing, and that their significance is probably of little consequence, but further investigation should be of interest.